

METHODS AND COMPOSITIONS FOR NUCLEIC ACID DELIVERY

This application claims the benefit of U.S. provisional application number 60/240,231 filed on October 13, 2001, the disclosure of which application is incorporated herein by reference.

5 STATEMENT OF U.S. GOVERNMENT SUPPORT

Funding for the present invention was provided in part by the Government of the United States by virtue of the National Institutes of Health grant. Accordingly, the Government of the United States has certain rights in this invention.

10 BACKGROUND OF THE INVENTION

1. Field of the invention.

The present invention relates to improved gene transfer methods and, more particularly, methods that enable highly efficient and widespread delivery of selected nucleic acids, to solid organs such as the heart or liver as well as to other solid cell
15 masses such as a solid tumor. Preferred methods of the invention include treatment of tissue and/or cells for delivery of nucleic acid with one or more phosphodiesterase inhibitor compounds such as sildenafil.

2. Background.

20 Effective delivery of nucleic acid to cells or tissue with high levels of expression are continued goals of gene transfer technology. As a consequence of the general inability to achieve those goals to date, however, clinical use of gene transfer methods has been limited.

25 Thus, for example, several delivery schemes have been explored for *in vivo* myocardial gene transfer, but none has proven capable of modifying a majority of cardiac myocytes in a homogeneous fashion. Techniques involving injection directly

into the myocardium are considered of limited use because gene expression does not extend significantly beyond the needle track. R. J. Guzman et al. *Circ Res* 1993; 73:1202-1207; A. Kass-Eisler *Proc Natl Acad Sci* 1993; 90:11498-11502. In one study, percutaneous intracoronary delivery of 10^{10} pfu of adenovirus caused infection in only
5 about one-third of the myocytes in the region served by the target artery. E. Barr et al. *Gene Therapy* 1994, 1:51-58.

Other coronary delivery models, either *in situ* or *ex vivo*, have produced a very small percentage of infected cells spread throughout the heart. J. Muhlhauser et al.
10 *Gene Therapy* 1996; 3:145-153; J. Wang et al. *Transplantation* 1996; 61:1726-1729. To date, no *in vivo* delivery system has been able to infect a majority of cells in an intact heart.

Certain gene delivery procedures also have been quite invasive and hence undesirable. For example, one report describes essentially complete loss of
15 endothelium by mechanical or proteolytic means to enable gene transfer from blood vessels to cells positioned across interposing endothelial layers. See WO 93/00051.

Certain gene transfer applications also have been explored in other organs such as the liver. In particular, *ex vivo* strategies have included surgical removal of
20 selected liver cells, genetic transfer to the cells in culture and then reimplantation of the transformed cells. See M. Grossman et al., *Nat Genet* 1994, 6:335-341. Such an *ex vivo* approach, however, suffers from a number of drawbacks including, for example, the required hepatocyte transplantation. M.A. Kay et al., *Science* 1993, 262:117-119; and S.E. Raper et al., *Cell Transplant* 1993, 2:381-400. *In vivo* strategies for gene
25 transfer to the liver also have been investigated, but have suffered from low delivery efficiencies as well as low specificity to the targeted tissue. N. Ferry et al., *Proc Natl Acad Sci USA* 1991, 88:8377-8391; A. Lieber et al. *Proc Natl Acad Sci USA* 1995, 6:6230-6214; A.L. Vahrmeijer et al., *Reg Cancer Treat* 1995, 8:25-31. See also P. Heikkilä et al., *Gene Ther* 1996, 3(1):21-27.

Gene transfer has been generally unsuccessful in additional applications. For example, gene transfer therapies for treatment of cystic fibrosis have largely failed because transduction of insufficient numbers of cells.

It thus would be desirable to have improved methods and systems to
5 effectively deliver nucleic acid to targeted cells and tissue. It would be particularly desirable to have new methods and systems for effective delivery of nucleic acids into solid organs, especially the heart, liver, lung and the like, as well as other solid cell masses such as a solid tumor.

10 SUMMARY OF THE INVENTION

We have now found methods and compositions that enable effective delivery of nucleic acids to desired cells, including to a solid mass of cells, particularly a solid organ such as a mammalian heart, liver, kidney, skeletal muscle, spleen or prostate, or to malignant cells such as a solid tumor. These methods and compositions enable
15 effective gene transfer and subsequent expression of a desired gene product to a majority of cells throughout a solid cell mass, and/or gene transfer and subsequent expression of a desired gene product to a solid cell mass.

We have found that administration of one or more PDE inhibitor compounds,
20 particularly a PDE-5 inhibitor compound such as sildenafil can significantly enhance nucleic acid delivery to targeted tissue.

Without being bound by any theory, we believe that administration of a PDE-5 inhibitor compound can amplify endogenous cGMP thereby causing an increase in
25 vascular permeability.

We also have found particularly enhanced nucleic acid delivery results from combined administration of a PDE-5 inhibitor compound such as sildenafil and a further distinct permeability enhancement agent such as VEGF. Accordingly, such a
30 "cocktail" of a PDE inhibitor compound and an additional, distinct permeability enhancement agent such as VEGF is particularly preferred.

Methods and compositions of the invention preferably provide enhanced vascular permeability that enables increased nucleic acid delivery to targeted cells. In particular, methods of the invention provide for treatment of treated cells with one or
5 more phosphodiesterase inhibitor compounds. Particularly preferred permeability enhancement compounds include sildenafil, zaprinast and T-1032 (Tanabe Seiyaku Co.).

Examples of particular phosphodiesterase (PDE) inhibitors have been
10 previously reported in U.S. Pat. Nos. 6,100,270; 6,006,735; 6,143,757; 6,143,746; 6,140,329; 6,117,881; 6,043,252; 6,001,847; 5,981,527; and 6,207,829 B1; the disclosures of which patents are incorporated herein by reference. See also PCT/EP95/04065; WO-A-93/06104; WO-A-93/07149; WO-A-93/12095; WO-A-94/00453; EP 0 463756 B1; and WO-A-94/05661 for additional compounds. See
15 also U.S. Pat Nos. 4,753,945; 5,010,086; 6,121,279; 6,156,753; 6,054,475; 5,091,431; 6,127,363 and 6,040,309 for additional compounds useful as nucleic acid delivery agents in accordance with the invention. Additional PDE inhibitor compounds for use in accordance with the invention are disclosed in Komasa et al., *Phosphodiesterase Inhibitors* (1996) (Schudt eds.), Academic Press, San Diego, CA.

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Preferred PDE inhibitors for use with the invention include, but are not limited to, particular bicyclic heterocyclic PDE inhibitors, more preferably pyrazolo[4,3-d]pyrimidin-7-ones, pyrazolo[3,4-d]pyrimidin-4-ones, quinazolin-4-ones, purin-6-ones, pyrido[3,2-d]pyrimidin-4-ones; as well as pharmaceutically acceptable salts thereof.

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A specifically preferred pyrazolo[4,3-d]pyrimidin-7-one is sildenafil (ViagraTM), also known as 5-[2-ethoxy-5-(4-methylpiperazin-1-ylsulphonyl)phenyl]-1-methyl-3-n-propyl-1,6-dihydro-7H-pyrazolo[4,3d]pyrimidin-7-one; as well as pharmaceutically acceptable salts thereof.

30

As demonstrated in the example which follow, use of a PDE inhibitor compound can significantly enhances transfer of administered nucleic acid to targeted cells. A PDE inhibitor compound suitably may be administered through the vasculature of targeted tissue prior to administration of nucleic acid, and/or the PDE inhibitor compound and exogenous nucleic acid can be administered simultaneously. Preferably, the vasculature of targeted tissue is pre-treated with a PDE inhibitor agent.

In preferred aspects of the invention, a vasculature permeability agent in addition to a PDE inhibitor is administered to tissue or cells to be treated with exogenous nucleic acid. Suitable additional, distinct permeability agents include e.g. serotonin and bradykinin. Other suitable additional, distinct permeability agents will include platelet-activating factor (PAF), prostaglandin E₁ (PGE₁), histamine, vascular endothelium growth factor (VEGF), zona occludens toxin (ZOT), interleukin-2 and other plasma kinins in addition to bradykinin. Nitric oxide agonists or promoters (activators) such as nitroglycerin or nitroprusside. Additional nitric oxide synthase activators can be readily identified by known in vitro assays that detect increases in nitric oxide activity. References herein to a nitric oxide synthase activator or nitric oxide activator or other similar term refers to compounds that activate nitric oxide synthase (e.g. at least about 10%, 20%, 30%, 40% or 50% increase relative to a control) or in such an in vitro assay.

Also preferred permeability agents that can increase cyclic guanosine 3'-monophosphate (cGMP), such as determined by a radioimmunoassay, including such assays commercially available from Amersham Pharmacia Biotech. References herein to a cGMP activator or other similar term refers to compounds that provide increased (e.g. at least 20%, 30%, 40%, or 50% increase relative to a control) in such an in vitro assay.

Other suitable vasculature permeability agents can be readily identified, e.g. simply by testing a candidate permeability agent to determine if it enhances uptake of

nucleic acid by targeted tissue relative to a control tissue sample that has not been exposed to the candidate permeability agent.

5 A single or a combination of more than one distinct permeability agents may be administered in a particular application. In this regard, a particular application can be optimized by selection of an optimal permeability agent, or optimal "cocktail" of multiple permeability agents. Such optional agent(s) can be readily identified by those skilled in the art by routine procedures, e.g. testing selected permeability agents and combinations thereof in *in vivo* assays.

10

Low extracellular calcium ion concentration conditions also can be used to enhance vascular permeability. It has been found that transfer of administered nucleic acid to targeted cells is substantially enhanced under such conditions. Low calcium concentration conditions may be readily provided, particularly by perfusing a low calcium ion concentration fluid through the vasculature of the tissue to which nucleic acid is administered. Suitable perfusate calcium ion concentrations may be about 500 $\mu\text{mol/L}$ or less, e.g. within a range from about 40 or 50 $\mu\text{mol/L}$ to about 500 $\mu\text{mol/L}$, more preferably from about 50 $\mu\text{mol/L}$ to about 200 $\mu\text{mol/L}$. A perfusate calcium concentration of about 50 $\mu\text{mol/L}$ is particularly preferred. Calcium ion (e.g. Ca^{2+}) concentration also can be lowered through use of a suitable buffer such as a chelating agent, e.g. ethylenedis(oxyethylenenitrilo)tetracetic acid (EGTA), ethylenediaminetetracetic acid (EDTA), or 1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA).

25 Administration conditions in addition to those discussed above also will impact efficiency of nucleic acid uptake by targeted tissue. In particular, concentration, amount and exposure time of the administered nucleic acid and temperature of the targeted tissue all can effect the rate of gene transfer. Flow rate and perfusion pressure also can effect the rate of gene transfer.

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More specifically, in a perfusion administration protocol, the rate of gene transfer increases with increase in concentration, total amount and exposure time of the administered nucleic acid in a perfusate. High concentration of nucleic acid in a perfusate especially can increase gene transfer rate. Preferred perfusate concentrations can vary with a number of factors including the particular organ or cell mass being treated, the particular cloning vehicle being administered and the like. However, in general, preferred concentrations of a viral vector in an administered perfusate are about 1×10^8 pfu/ml or greater, more preferably a concentration of about 5×10^8 pfu/ml or greater. Administered perfusate also preferably may be recirculated and readministered to a subject, e.g. to limit the total viral burden introduced into the target, or if the administered agent is in short supply. However, effective gene transfer can be achieved without recirculation, particularly if other delivery parameters are optimized. Increases in either flow rate of perfusate pressure generally will increase gene transfer efficiency, although for clinical safety it can be desirable to limit both perfusate pressure and flow rate.

The rate of gene transfer also decreases with decreased temperature of targeted tissue, particularly where the tissue is below about 20°C . Delivery of the nucleic acid near body temperature of the subject is preferred, e.g. where the nucleic acid is administered at a temperature of from about $28-45^\circ\text{C}$, more preferably from about $34-40^\circ\text{C}$. However, gene transfer can be achieved over wide temperature ranges, e.g. at about 4°C . Clinical circumstances may require lower temperatures, e.g. with gene transfer during cardiac surgery.

Nucleic acid administered in accordance with the invention can express a desired therapeutic agent, or may inhibit expression or function of an endogenous gene of a subject. Nucleic acid also may be administered for diagnostic purposes, for example to express a marker protein. In addition to such therapeutic and diagnostic methods, methods and compositions of the invention also may be employed to examine the effect of a heterologous gene on an intact organ such as a subject's heart,

to create animal models of disease and to provide mechanistic information regarding various disease states.

In a preferred aspect, the invention includes methods for xenotransplantation. Thus, for example, cells of xenogeneic tissue, particularly cells of a xenogeneic solid cells mass, can be administered exogenous nucleic acid under enhanced vascular permeability as described herein. Those cells containing exogenous nucleic acid then may be transplanted into a subject. More particularly, the exogenous nucleic acid can be administered *in vivo* or *ex vivo* to donors cells or organs, e.g. a xenogeneic heart, liver, spleen and the like, in accordance with the invention and the donor cells or organ can be transplanted to a selected host, e.g. a mammal, particularly a primate such as a human. Suitable donor organs may be obtained from e.g. another primate, or a swine, particularly a pig. A variety of exogenous nucleic acids can be administered to the donor cells. For instance, nucleic acids can be administered that will express a gene product that can promote a desired phenotypic characteristic. Exemplary gene products include those which can reduce immune system recognition of the xenotransplanted cells.

In another aspect, the invention provides methods and compositions for enhancing nucleic acid delivery to targeted cells by use a vasculature permeability enhancement that is a promotes nitric oxide synthase or camp, such as nitroglycerin or nitroprusside, i.e. alone without co-administration with a PDE inhibitor compound.

In another aspect, the invention includes vasculature permeability and gene transfer solutions useful in the methods of the invention. Such solutions in general will be formulated to provide enhanced permeability to treated tissue upon administration of nucleic acid to such layers. Thus, suitable permeability agents will include one or more permeability agents as disclosed herein, particularly at least one PDE inhibitor compounds, and optionally one or more additional, distinct permeability agents, and optionally low calcium ion concentrations (e.g. about 500 $\mu\text{mol/L}$ or less). A solution of the invention also may contain one or more therapeutic agents, e.g. one or more exogenous nucleic acids (e.g. one or more

recombinant adenoviruses) to be administered to a subject, or other pharmaceutical agent such as nitroglycerine to control vasospasms and the like to a solution that will be administered to a heart. A permeability or gene transfer solution may suitably contain nucleic acid in a form for administration e.g. in a suitable cloning vehicle such as a viral vector dissolved in desired pharmaceutically acceptable carrier e.g. Krebs solution or other buffered solution. A permeability or gene transfer solution of the invention preferably will be pharmaceutically acceptable, e.g. sterile and otherwise suitable for administration to a subject. Typically a vasculature permeability or gene transfer solution will be stored in a sealed (preferably, hermetically sealed) container prior to use. A permeability or gene transfer solution preferably will contain active ingredients (i.e., permeability agent, calcium ion concentration, nucleic acid) in optimal dosage quantities. Solutions of the invention may suitably have other agents such as various buffers, sugars, salts and the like.

A wide variety of cells may be treated in accordance with the invention. Suitable cells for administration include those that have a distinct circulation, or circulation that can be isolated in some manner. Thus, for example, organs and other cell masses are suitable for administration in accordance with the invention including e.g. heart, lung, liver, kidney, prostate, testes, ovaries, skeletal muscle, kidneys, brain, spleen and solid tumors. Exemplary tumors that can be treated in accordance with the invention include e.g. cancers of the lung, prostate, liver, brain, testes or ovaries. Cells treated in accordance with the invention may be in either a healthy or diseased state. A wide variety of subjects also may be treated in accordance with the invention. Typical subjects include mammals, particularly primates, especially humans.

Other aspects of the invention are discussed below.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1A shows results of Example 1, particularly enhanced gene transfer with a PDE inhibitor use (Substance P).

Fig 2 includes Fig 2(a) and 2(b). Fig 2(a) shows a schematic of intracellular pathways mediating increase in vascular permeability and gene transfer. Fig. 2(b) shows the effect of perfusion with nitroglycerin (TNG) or nitroprusside (SNP) or with 5-Br-cGMP on adenovirus-mediated gene transfer. TNG and SNP increase intracellular NO, and 5-Br-cGMP intracellular cGMP. For Fig. 2(a), abbreviations are PL-C γ : phospholipase C- γ ; NOS: nitric acid synthase; PDE-5, phosphodiesterase 5; GC: guanylate cyclase; PK-6: protein kinase G.

Fig. 3 shows results of the example, particularly gene transfer. Ex vivo perfused hearts were exposed to VEGF (0.3×10^{-10} M, 2 minutes) alone or after 15 minutes exposure to the PDE-5 inhibitors of sildenafil (1×10^{-5} M), zaprinast (1×10^{-5} M) or T-1032 (1×10^{-6} M). Hearts were then exposed to Ad β gal (1×10^{-8} pfu/ml, 2 min), and the percentage of cells receiving the transgene quantified. n = 3 for each except n = 1 for T-1032.

DETAILED DESCRIPTION OF THE INVENTION

As stated above, the present invention provides methods and compositions that enable effective delivery of nucleic acid to desired cells, including to cells of a solid cell mass, e.g. of an organ such as a mammalian heart or liver, or other solid cell mass such as a solid tumor.

We have found that administration of one or more PDE inhibitor compounds, particularly a PDE-5 inhibitor compound such as sildenafil can significantly enhance nucleic acid delivery to targeted tissue.

In additional aspects, the invention includes methods and compositions as disclosed herein that include use or administration or inclusion of Substance P, nitroglycerin, nitroprusside (such as sodium nitroprusside), 8-Br-cGMP, zaprinast and T-1032 as the sole agent for enhancing nucleic acid delivery, or in combination with

one or more other agents as disclosed herein such as sildenafil or other PDE-5 inhibitor compound, or other agent such as VEGF.

Preferably, the methods and compositions of the invention provide enhanced
5 microvascular permeability that enables increased gene transfer to targeted cells. As discussed above, these methods and compositions create transient permeability of endothelial layers and without significant degradation or injury to endothelial cell layers of a solid cell mass.

10 As used herein, the term "without significant degradation or injury to endothelial cell layers" or other similar phrase means that less than 25%, more typically less than 15 or 10 percent, of endothelial cells are lost from the treated vessel wall prior to administration of nucleic acid to a subject. This contrasts from
15 denuding and consequent injury of the endothelium through mechanical trauma or use of proteolytic enzymes which would result through use of prior methods. Lack of significant degradation of endothelial cell layers can be readily determined, e.g. by microscopic examination of the layer. Also, lack of significant degradation of endothelial cell layers can be determined by simple testing. For example, an
20 endothelial layer can be tested to determine whether the layer has maintained a particular function and therefore is significantly degraded, e.g. a layer's ability to exhibit a reversible vasodilator response to 10^{-7} mol/L acetylcholine.

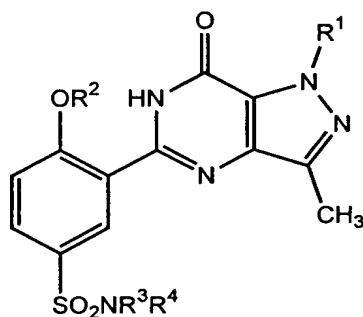
In addition to the above discussed PDE inhibitor compounds, suitable PDE
inhibitor compounds for use in the methods of the invention are disclosed below,
25 include compounds of the following Formulae I to XIII, which are generally preferred for use with the present invention. It should be appreciated however that the present invention is not limited by any particular PDE inhibitor compound, and the invention is applicable to any such PDE inhibitor compound now known or subsequently
discovered or developed.

In general, PDE-5 inhibitor compounds are preferred for use in the methods and compositions of the invention.

More specifically, in one invention embodiment, at least one of the
5 administered compounds is a bicyclic heterocyclic PDE inhibitor such as described in the U.S. Pat. No. 6,100,270, preferably at least one of the following pyrazolo[4,3-d]pyrimidin-7-ones, pyrazolo[3,4-d]pyrimidin-4-ones, a quinazolin-4-ones, a purin-6-ones, or pyrido[3,2-d]pyrimidin-4-ones set forth in the following Formulae I-V including pharmaceutically acceptable salts thereof.

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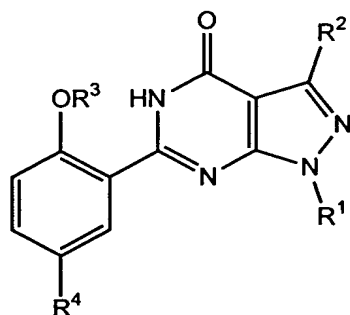
Suitable PDE inhibitor compounds include those of the following Formula I:



(I)

15 wherein in Formula I, R¹ is methyl or ethyl; R² is ethyl or n-propyl; and R³ and R⁴ are each independently H, or C₁-C₆ alkyl optionally substituted with C₃-C₇ cycloalkyl or with morpholino; and pharmaceutically acceptable salts thereof.

Suitable PDE inhibitor compounds also include those of the following
20 Formula II:



(II)

wherein in Formula II is C₁ -C₆ alkyl; R² is H; methyl or ethyl;

R³ is C₂ -C₄ alkyl;

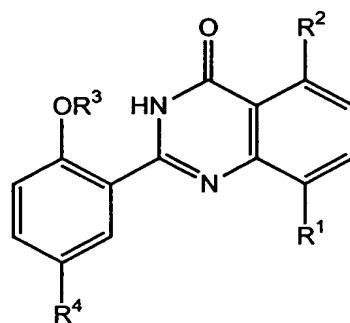
5 R⁴ is H; C₁ -C₄ alkyl optionally substituted with NR⁵ R⁶, CN, CONR⁵ R⁶ or CO₂ R⁷; C₂ -C₄ alkenyl optionally substituted with CN, CONR⁵ R⁶ or CO₂ R⁷; C₂ -C₄ alkanoyl optionally substituted with NR⁵ R⁶; SO₂ NR⁵ R⁶; CONR⁵ R⁶; CO₂ R⁷ or halo;

10 R⁵ and R⁶ are each independently H or C₁ -C₄ alkyl; or, together with the nitrogen atom to which they are attached, form a pyrrolidino, piperidino, morpholino, 4-(NR⁸)-1-piperazinyl or 1-imidazolyl group wherein said group is optionally substituted with one or two C₁ -C₄ alkyl groups;

R⁷ is H or C₁ -C₄ alkyl;

15 and R⁸ is H; C₁ -C₃ alkyl or (hydroxy)C₂ -C₃ alkyl; and pharmaceutically salts thereof.

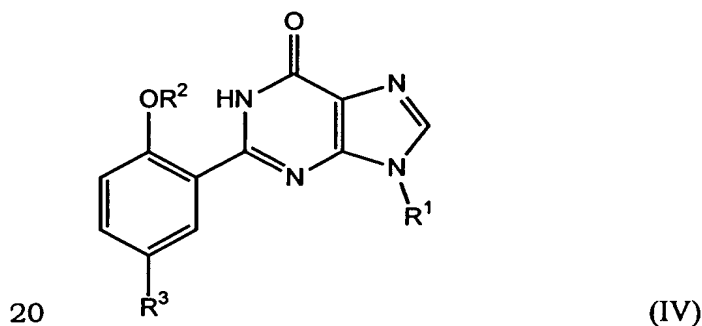
Additional suitable PDE inhibitor compounds include those of the following Formula (III):



(III)

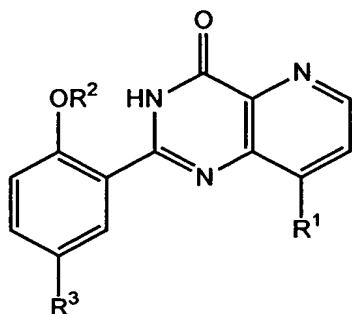
- wherein in Formula III R^1 is H; C_1 - C_4 alkyl; C_1 - C_4 alkoxy or $CONR^5 R^6$;
 R^2 is H or C_1 - C_4 alkyl;
 R^3 is C_2 - C_4 alkyl;
 R^4 is H; C_2 - C_4 alkanoyl optionally substituted with $NR^7 R^8$; (hydroxy) C_2 - C_4 alkyl
5 optionally substituted with $NR^7 R^8$; $CH=CHCO_2 R^9$; $CH=CHCONR^7 R^8$; $CH_2 CH_2$
 $CO_2 R^9$; $CH_2 CH_2 CONR^7 R^8$; $SO_2 NR^7 R^8$; $SO_2 NH(CH_2)_n NR^7 R^8$ or imidazolyl;
 R^5 and R^6 are each independently H or C_1 - C_4 alkyl;
 R^7 and R^8 are each independently H or C_1 - C_4 alkyl; or, together with the nitrogen
atom to which they are attached, form a pyrrolidino, piperidino, morpholino or 4-
10 (NR^{10}) -1-piperazinyl group wherein any of said groups is optionally substituted with
 $CONR^5 R^6$;
 R^9 is H or C_1 - C_4 alkyl;
 R^{10} is H; C_1 - C_3 alkyl or (hydroxy) C_2 - C_3 alkyl;
and n is 2, 3 or 4;
15 with the proviso that R^4 is not H when R^1 is H, C_1 - C_4 alkyl or C_1 - C_4 alkoxy; and
pharmaceutically acceptable salts thereof.

Suitable PDE inhibitor compounds include those of the following Formula IV:



- wherein R^1 is C_1 - C_4 alkyl; R^2 is C_2 - C_4 alkyl;
 R^3 is H or $SO_2 NR^4 R^5$;
 R^4 and R^5 together with the nitrogen atom to which they are attached form a
25 pyrrolidino, piperidino, morpholino or 4- (NR^6) -1-piperazinyl group;
and R^6 is H or C_1 - C_3 alkyl; and pharmaceutically acceptable salts thereof.

Additional suitable PDE inhibitor compounds include those of the following Formula (V):



5 (V)

wherein in Formula V R¹ is H; C₁-C₄ alkyl; CN or CONR⁴ R⁵ ; R² is C₂-C₄ alkyl;

R³ is SO₂ NR⁶ R⁷ ; NO₂ ; NH₂ ; NHCOR⁸ ; NHSO₂ R⁸ or N(SO₂ R⁸)₂ ;

R⁴ and R⁵ are each independently selected from H and C₁-C₄ alkyl;

10 R⁶ and R⁷ are each independently selected from H and C₁-C₄ alkyl optionally substituted with CO₂ R⁹, OH, pyridyl, 5-isoxazolin-3-onyl, morpholino or 1-imidazolidin-2-onyl; or, together with the nitrogen atom to which they are attached, form a pyrrolidino, piperidino, morpholino, 1-pyrazolyl or 4-(NR¹⁰)-1-piperazinyl group wherein any of said groups may optionally be substituted with one or two

15 substituents selected from C₁-C₄ alkyl, CO₂ R⁹, NH₂ and OH;

R⁸ is C₁-C₄ alkyl or pyridyl;

R⁹ is H or C₁-C₄ alkyl;

and R¹⁰ is H; C₁-C₄ alkyl or (hydroxy)C₂-C₃ alkyl; and a pharmaceutically acceptable salt thereof

20

A preferred group of compounds of Formula I above include those wherein:

R³ is H; methyl or ethyl;

R⁴ is C₁-C₆ alkyl optionally substituted with cyclohexyl or with morpholino; and

R¹ and R² are as previously defined for formula (I), and pharmaceutically acceptable

25 salts thereof.

Preferred compounds of Formula II above include those wherein R¹ is n-propyl; R² is H or methyl; R³ is ethyl or n-propyl; R⁴ is H; ethyl substituted with CONR⁵R⁶ or CO₂R⁷; vinyl substituted with CONR⁵R⁶ or CO₂R⁷; acetyl substituted with NR⁵R⁶; SO₂NR⁵R⁶; CONR⁵R⁶; CO₂R⁷ or bromo; R⁵ and R⁶ together with the nitrogen atom to which they are attached form a morpholino, 4-(NR⁸)-1-piperazinyl or 2,4-dimethyl-1-imidazolyl group; R⁷ is H or t-butyl; and R⁸ is methyl or 2-hydroxyethyl; and pharmaceutically acceptable salts thereof.

Preferred compounds of Formula III above include those where R¹ is H; methyl; methoxy or CONR⁵R⁶; R² is H or methyl; R³ is ethyl or n-propyl; R⁴ is H; acetyl optionally substituted with NR⁷R⁸; hydroxyethyl substituted with NR⁷R⁸; CH=CHCO₂R⁹; CH=CHCONR⁷R⁸; CH₂CH₂CO₂R⁹; SO₂NR⁷R⁸; SO₂NH(CH₂)₃NR⁷R⁸ or 1-imidazolyl; R⁵ and R⁶ are each independently H or ethyl; R⁷ and R⁸ together with the nitrogen atom to which they are attached form a piperidino, 4-carbamoylpiperidino, morpholino or 4-(NR¹⁰)-1-piperazinyl group; R⁹ is H or t-butyl; and R¹⁰ is H; methyl or 2-hydroxyethyl; with the proviso that R⁴ is not H when R¹ is H, methyl or methoxy; and pharmaceutically acceptable salts thereof.

Preferred compounds of Formula IV above include those wherein R¹ and R² are each independently ethyl or n-propyl; R⁴ and R⁵ together with the nitrogen atom to which they are attached form a 4-(NR⁶)-1-piperazinyl group; and R³ and R⁶ are as previously defined for Formula IV; and pharmaceutically acceptable salts thereof.

Preferred compounds of Formula V above include compounds wherein R¹ is H; n-propyl; CN or CONH₂; R² is ethyl; R³ is SO₂NR⁶R⁷; NO₂; NH₂; NHCOCH(CH₃)₂; NHSO₂CH(CH₃)₂; NHSO₂(3-pyridyl) or N[SO₂(3-pyridyl)]₂; R⁶ is H; methyl or 2-hydroxyethyl; R⁷ is methyl optionally substituted with 2-pyridyl or 5-isoxazolin-3-onyl; or ethyl 2-substituted with OH, CO₂CH₂CH₃, morpholino or 1-imidazolidin-2-onyl; or R⁶ and R⁷ together with the nitrogen atom to which they are attached form a (4-CO₂R⁹)piperidino, 5-amino-3-hydroxy-1-pyrazolyl or 4-(NR¹⁰)-1-piperazinyl group; R⁹ is H or ethyl; and R¹⁰ is H; methyl or 2-hydroxyethyl.

A particularly preferred group of compounds is that of Formula III above wherein R^1 is methyl; CONH_2 or $\text{CONHCH}_2\text{CH}_3$; R^2 is H; R^3 is ethyl or n-propyl; R^4 is H; acetyl; 1-hydroxy-2-(NR^7R^8)ethyl; $\text{CH}=\text{CHCO}_2\text{C}(\text{CH}_3)_3$; $\text{CH}=\text{CHCONR}^7$
5 R^8 ; $\text{SO}_2\text{NR}^7\text{R}^8$ or 1-imidazolyl, R^7 and R^8 together with the nitrogen atom to which they are attached form a 4-(NR^{10})-1-piperazinyl group; and R^{10} is methyl or 2-hydroxyethyl; with the proviso that R^4 is not H when R^1 is methyl; of formula (IV) wherein R^1 is n-propyl; R^2 is ethyl; and R^3 is 1-piperazinylsulphonyl or 4-methyl-1-piperazinylsulphonyl; and of formula (V) wherein R^1 is n-propyl or CN; R^2 is ethyl;
10 R^3 is $\text{SO}_2\text{NR}^6\text{R}^7$; $\text{NHSO}_2\text{CH}(\text{CH}_3)_2$; NHSO_2 (3-pyridyl) or $\text{N}[\text{SO}_2$ (3-pyridyl)]₂; R^6 is H or methyl; R^7 is methyl; or ethyl 2-substituted with $\text{CO}_2\text{CH}_2\text{CH}_3$; morpholino or 1-imidazolidin-2-onyl; or R^6 and R^7 together with the nitrogen atom to which they are attached form a (4- CO_2R^9)piperidino or 4-(NR^{10})-1-piperazinyl group; R^9 is H or ethyl; and R^{10} is H; methyl or 2-hydroxyethyl.

15

Especially preferred individual compounds of the invention include:

- 1-ethyl-5-[5-(n-hexylsulphamoyl)-2-n-propoxy-phenyl]-3-methyl-1,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one;
- 20 1-ethyl-5-(5-diethylsulphamoyl-2-n-propoxy-phenyl)-3-methyl-1,6-dihydro-7H-pyrazolo[4,3-d]-pyrimidin-7-one;
- 25 5-[5-(N-cyclohexylmethyl-N-methylsulphamoyl)-2-n-propoxyphenyl]-1-ethyl-3-methyl-1,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one;
- 6-(5-bromo-2-n-propoxyphenyl)-3-methyl-1-n-propyl-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one;
- 30 3-methyl-6-(5-morpholinosulphonyl-2-n-propoxyphenyl)-1-n-propyl-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one;

6-[5-(2-carboxyvinyl)-2-n-propoxyphenyl]-3-methyl-1-n-propyl-1,5-dihydro-4H -
pyrazolo[3,4-d]pyrimidin-4-one;

- 5 6-[5-(2-t-butoxycarbonylvinyl)-2-n-propoxyphenyl]-3-methyl-1-n-propyl-1,5-
dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one;

3-methyl-6-[5-(2-morpholinocarbonylvinyl)-2-n-propoxyphenyl]-1-n-propyl-1,5 -
dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one;

10

3-methyl-6-[5-(2-morpholinocarbonylethyl)-2-n-propoxyphenyl]-1-n-propyl-1,5-
dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one;

15

2-{2-ethoxy-5-[4-(2-hydroxyethyl)-1-piperazinylsulphonyl]phenyl}-8-
methylquinazolin-4-(3H)-one;

2-{5-[4-(2-hydroxyethyl)-1-piperazinylsulphonyl]-2-n-propoxyphenyl}-8-
methylquinazolin-4(3H)-one;

20

8-methyl-2-{5-[2-(4-methyl-1-piperazinylcarbonyl)-ethenyl]-2-n-
propoxyphenyl}quinazolin-4(3H)-one;

8-carbamoyl-2-{2-ethoxy-5-[4-(2-hydroxyethyl)-1-
piperazinylsulphonyl]phenyl}quinazolin-4(3H)-one;

25

8-ethylcarbamoyl-2-(2-n-propoxyphenyl)quinazolin-4(3H)-one;

2-[2-ethoxy-5-(4-ethoxycarbonylpiperidino-sulphonyl)phenyl]-8-n-propylpyrid o[3,2-
d]pyrimidin-4(3H)-one;

30

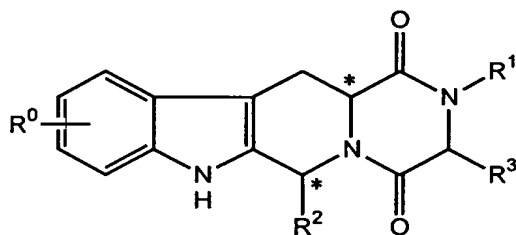
2-[5-(4-carboxypiperidinosulphonyl)-2-ethoxyphenyl]-8-n-propylpyrido[3,2-d]pyrimidin-4(3H)-one;

2-{2-ethoxy-5-[4-(2-hydroxyethyl)-1-piperazinyl-sulphonyl]phenyl}-8-n-propylpyrido[3,2-d]pyrimidin-4(3H)-one;

and 2-{2-ethoxy-5-[(bis-3-pyridylsulphonyl)amino]-phenyl}-8-n-propylpyrido[3,2-d]pyrimidin-4(3H)-one.

10 In another invention embodiment, at least one of the administered compounds is a tetracyclic cGMP specific PDE inhibitor such as those described in U.S. Pat. No. 6,143,746 and as set forth in the following Formulae VI-IX including pharmaceutically acceptable salts thereof.

15 More specifically, suitable compounds include those of the following Formula VI:

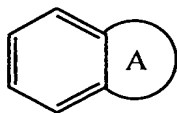


(VI)

20 wherein in Formula VI R⁰ represents hydrogen, halogen, or C₁₋₆ alkyl;

R¹ represents hydrogen, C₁₋₆ alkyl, C₂₋₆ alkenyl, C₂₋₆ alkynyl, haloC₁₋₆ alkyl, C₃₋₈ cycloalkyl, C₃₋₈ cycloalkylC₁₋₃ alkyl, arylC₁₋₃ alkyl, or heteroarylC₁₋₃ alkyl;

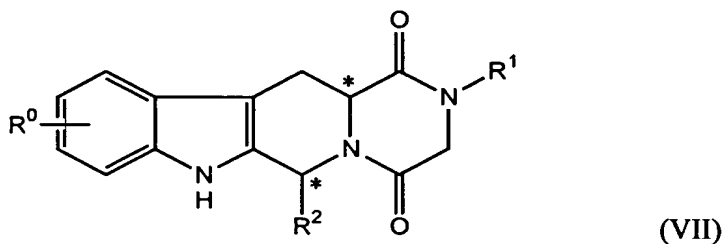
25 R² represents an optionally substituted monocyclic aromatic ring selected from benzene, thiophene, furan, and pyridine, or an optionally substituted bicyclic ring;



attached to the rest of the molecule via one of the benzene ring carbon atoms and wherein the fused ring A is a 5- or 6-membered ring which may be saturated or partially or fully unsaturated and comprises carbon atoms and optionally one or two heteroatoms selected from oxygen, sulphur, and nitrogen; and

R^3 represents hydrogen of C_{1-3} alkyl, or R^1 and R^3 together represent a 3- or 4-membered alkyl or alkenyl chain; and pharmaceutically and salts and solvates (e.g., hydrates) thereof.

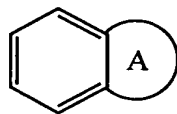
Suitable compounds also include those of the following Formula VII:



wherein in Formula VII R^0 represents hydrogen, halogen, or C_{1-6} alkyl;

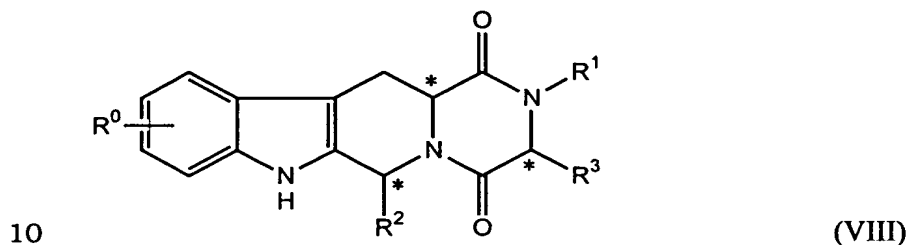
R^1 represents hydrogen, C_{1-6} alkyl, halo C_{1-6} alkyl, C_3-8 cycloalkyl C_{1-3} alkyl, aryl C_{1-3} alkyl, or heteroaryl C_{1-3} alkyl; and

R^2 represents an optionally substituted monocyclic aromatic ring selected from benzene, thiophene, furan, and pyridine, or an optionally substituted bicyclic ring



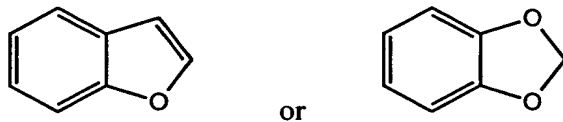
attached to the rest of the molecule via one of the benzene ring carbon atoms, and wherein the fused ring A is a 5- or 6-membered ring which can be saturated or partially or fully unsaturated and comprises carbon atoms and optionally one or two heteroatoms selected from oxygen, sulphur, and nitrogen; and pharmaceutically acceptable salts and solvates (e.g., hydrates) thereof.

A further subgroup of compounds of Formula VI preferred for use in the methods of the invention, are compounds of the following Formula VIII:



wherein in Formula VIII:

- 15 R^0 represents hydrogen, halogen, or C_{1-6} alkyl;
 R^1 represents hydrogen or C_{1-6} alkyl;
 R^2 represents the bicyclic ring



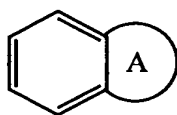
- 20 which can be optionally substituted by one or more groups selected from halogen and C_{1-3} alkyl; and

R^3 represents hydrogen or C_{1-3} alkyl; and pharmaceutically acceptable salts and solvates (e.g., hydrates) thereof.

In Formula VII above, with respect to R^1 , the term "aryl" as part of an arylC₁₋₃ alkyl group means phenyl or phenyl substituted by one or more (e.g., 1, 2, or 3) substituents selected from halogen, C₁₋₆ alkyl, C₁₋₆ alkoxy, and methylenedioxy. The term "heteroaryl" as part of a heteroarylC₁₋₃ alkyl group means thienyl, furyl, or pyridyl, each optionally substituted by one or more (e.g., 1, 2, or 3) substituents selected from halogen, C₁₋₆ alkyl, and C₁₋₆ alkoxy. The term "C₃-8 cycloalkyl" as a group or part of a C₃-8 cycloalkylC₁₋₃ alkyl group means a monocyclic ring comprising three to eight carbon atoms. Examples of suitable cycloalkyl rings include the C₃-6 cycloalkyl rings cyclopropyl, cyclobutyl, cyclopentyl, and cyclohexyl.

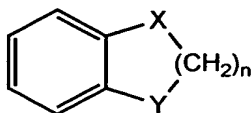
In formula VII above, with respect to R^2 , optional benzene ring substituents are selected from one or more (e.g., 1, 2, or 3) atoms or groups comprising halogen, hydroxy, C₁₋₆ alkyl, C₁₋₆ alkoxy, CO₂ R^b, haloC₁₋₆ alkyl, haloC₁₋₆ alkoxy, cyano, nitro, and NR^a R^b, where R^a and R^b are each hydrogen or C₁₋₆ alkyl, or R^a also can represent C₂₋₇ alkanoyl or C₁₋₆ alkylsulphonyl. Optional substituents for the remaining ring systems are selected from one or more (e.g., 1, 2, or 3 atoms or groups comprising halogen, C₁₋₆ alkyl, C₁₋₆ alkoxy, and arylC₁₋₃ alkyl as defined above. The bicyclic ring

20



can, for example, represent naphthalene, a heterocycle such as benzoxazole, benzothiazole, benzisoxazole, benzimidazole, quinoline, indole, benzothiophene, benzofuran, or

25



wherein n is an integer 1 or 2 and X and Y each can represent CH₂, O, S, or NH.

Unless otherwise indicated, in the above formulae, as well as other formulae
5 described herein, the term "alkyl," as a group or part of a group, means a straight
chain or, where available, a branched chain moiety containing the indicated number of
carbon atoms. For example, it can represent a C₁₋₄ alkyl function as represented by
methyl, ethyl, n-propyl, i-propyl, n-butyl, s-butyl, and t-butyl. The term "alkenyl" as
10 indicated number of carbon atoms, such as vinyl and allyl groups. The term "alkynyl"
as used herein includes straight chained and branched alkynyl groups containing the
indicated number of carbon atoms, suitably acetylene.

Unless other indicated, in the above formulae, as well as other formulae
15 disclosed herein, the term "halogen" herein means a fluorine, chlorine, bromine, or
iodine atom.

Unless other indicated, in the above formulae, as well as other formulae
disclosed herein, the term "haloC₁₋₆ alkyl" means an alkyl group as defined above
20 comprising one to six carbon atoms substituted at one or more carbon atoms by one or
more (e.g., 1, 2, or 3) halogen atoms. Similarly, a haloC₁₋₆ alkoxy group is a haloC₁₋₆
alkyl group as defined above linked to the R² benzene ring via an oxygen atom.
Examples of haloC₁₋₆ alkyl groups include trifluoromethyl and 2,2,2-trifluoroethyl. An
example of a haloC₁₋₆ alkoxy group is trifluoromethoxy. The term "C₂₋₇ alkanoyl"
25 means a C₁₋₆ alkanoyl group where the C₁₋₆ alkyl portion is as defined above. An
example of a suitable C₂₋₇ alkanoyl group is the C₂ alkanoyl group acetyl.

Unless other indicated, in the above formulae, as well as other formulae
disclosed herein, when R⁰ is a halogen atom or a C₁₋₆ alkyl group, this substituent can
30 be sited at any available position on the phenyl portion of the tetracyclic ring.
However, a particular site of attachment is the ring 10-position.

The compounds of Formula VI can contain two or more asymmetric centers, and, thus, can exist as enantiomers or diastereoisomers. In particular, in Formula VII above, two ring chiral centers are denoted with asterisks. It is to be understood that the invention includes both mixture and separate individual isomers of the compounds of Formula (VII).

The compounds of Formula VI also can exist in tautomeric forms, and the invention includes both mixtures and separate individual tautomers thereof.

10

A particular group of compounds for use in the methods of the invention are those compounds of Formula VI in which R^0 is hydrogen or halogen (e.g., fluorine), especially hydrogen.

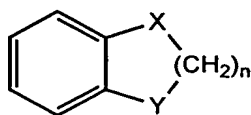
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Another particular group of compounds for use in the methods of the invention are those of Formula VI in which R^1 represents hydrogen, C_{1-4} alkyl, halo C_{1-4} alkyl, C_3-6 cycloalkyl, C_3-6 cycloalkylmethyl, pyridyl C_{1-3} alkyl, furyl C_{1-3} alkyl, or optionally substituted benzyl. Within this particular group of compounds, examples of C_{1-4} alkyl groups are methyl, ethyl, n-propyl, i-propyl, and n-butyl. Examples of C_3-6 cycloalkylmethyl groups are cyclopropylmethyl and cyclohexylmethyl. Examples of optionally substituted, benzyl groups include benzyl and halobenzyl (e.g., fluorobenzyl).

20

A further group of compounds for use in the methods of the invention are those compounds of Formula VI in which R^2 represents an optionally substituted benzene, thiophene, furan, pyridine, or naphthalene ring, or an optionally substituted bicyclic ring

25



Within the above definitions for Formula IX, R¹ preferably can represent C₁₋₄ alkyl (e.g., methyl, ethyl, i-propyl, and n-butyl), C₃₋₆ cycloalkyl (e.g., cyclopentyl) or C₃₋₆ cycloalkylmethyl (e.g., cyclopropylmethyl).

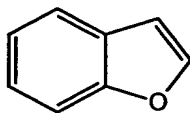
5 R² preferably can represent a substituted benzene ring such as benzene substituted by C₁₋₃ alkoxy (e.g., methoxy) or by C₁₋₃ alkoxy (e.g., methoxy) and halogen (e.g., chlorine), particularly 4-methoxyphenyl or 3-chloro-4-methoxyphenyl, or R² preferably can represent 3,4-methylenedioxyphenyl.

10 A particularly preferred subgroup of compounds of the above formula are compounds wherein R⁰ represents hydrogen.

A further preferred subgroup includes compounds wherein R¹ is selected from hydrogen, methyl, and isopropyl.

15

Preferably, R² represents the unsubstituted bicyclic ring



20 A still further subgroup of compounds of Formula IX, are compounds wherein R³ represents hydrogen or methyl.

It is to be understood that the present invention covers all appropriate combinations of particular and preferred groupings hereinabove.

25

Particular compounds suitable for use in the methods of the invention include:

cis-2,3,6,7,12,12a-hexahydro-2-(4-pyridyl-methyl)-6-(3,4-methylenedioxyphenyl)-pyrazino[2',1';6,1]-pyrido[3,4-b]indole-1,4-dione;

cis-2,3,6,7,12,12a-hexahydro-6-(2,3-dihydrobenzo[b]furan-5-yl)-2-methyl-
pyrazino[2',1';6,1]pyrido-[3,4-b]indole-1,4-dione;

- 5 cis-2,3,6,7,12,12a-hexahydro-6-(5-bromo-2-thienyl)-2-
methylpyrazino[2',1';6,1]pyrido[3,4-b]indole-1,4-dione;

cis-2,3,6,7,12,12a-hexahydro-2-butyl-6-(4-methylphenyl)-pyrazino[2',1';6,1]
pyrido[3,4-b]indole-1,4-dione;

10

(6R,12aR)-2,3,6,7,12,12a-hexahydro-2-isopropyl-6-(3,4-methylenedioxyphenyl) -
pyrazino[2',1';6,1]pyrido-[3,4-b]indole-1,4-dione;

15

(6R,12aR)-2,3,6,7,12,12a-hexahydro-2-cyclopentyl-6-(3,4-methylenedioxyphenyl)-
pyrazino[2',1';6,1]-pyrido[3,4-b]indole-1,4-dione;

(6R,12aR)-2,3,6,7,12,12a-hexahydro-2-cyclopropylmethyl-6-(4-methoxyphenyl)-
pyrazino[2',1';6,1]-pyrido[3,4-b]indole-1,4-dione;

20

(6R,12aR)-2,3,6,7,12,12a-hexahydro-6-(3-chloro-4-methoxyphenyl)-2-methyl-
pyrazino[2',1';6,1]pyrido[3,4-b]indole-1,4-dione;

(6R,12aR)-2,3,6,7,12,12a-hexahydro-2-methyl-6-(3,4-methylenedioxyphenyl)-
pyrazino[2',1';6,1]pyrido[3,4-b]indole-1,4-dione;

25

(6R,12aR)-2,3,6,7,12,12a-hexahydro-6-(3,4-methylenedioxyphenyl)-pyrazino[2',
1';6,1]pyrido[3,4-b]indole-1,4-dione;

(5aR, 12R, 14aS)-1,2,3,5,6,11,12,14a-octahydro-12-(3,4-methylenedioxyphenyl)-

30

pyrrolo[1'',2'':4'5']-pyrazino[2',1';6,1]pyrido[3,4-b]indole-5-1,4-dione;

(6R,12aR)-2,3,6,7,12,12a-hexahydro-6-(5-benzofuranyl)-2-methyl-pyrazino[2',1';6,1]pyrido[3,4-b]indole-1,4-dione;

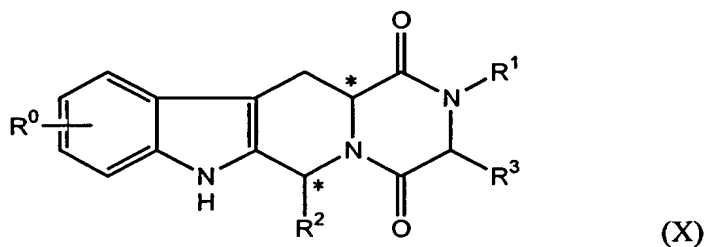
(6R,12aR)-2,3,6,7,12,12a-hexahydro-6-(5-benzofuranyl)-
5 pyrazino[2',1';6,1]pyrido[3,4-b]indole-1,4-dione;

(3S, 6R, 12aR)-2,3,6,7,12,12a-hexahydro-6-(5-benzofuranyl)-3-methyl-pyrazino[2',1';
6,1]pyrido[3,4-b]indole-1,4-dione;

10 (3S, 6R, 12aR)-2,3,6,7,12,12a-hexahydro-6-(5-benzofuranyl)-2,3-dimethyl-pyrazino[2',1';6,1]pyrido[3,4-b]indole-1,4-dione;

(6R, 12aR)-2,3,6,7,12,12a-hexahydro-6-(5-benzofuranyl)-2-isopropyl-pyrazino[2',
1',6,1]pyrido[3,4-b]indole-1,4-dione; and physiologically acceptable solvates (e.g.,
15 hydrates) thereof.

The invention is also compatible with administration of particular cGMP PDE inhibitors disclosed in U.S. Pat. No. 6,140,329, at least some of which compounds have been described in said U.S. Pat. No. 6,143,746. Preferred compounds of the
20 U.S. Patent No. 6,140,329 are set forth in the following Formula X including pharmaceutically acceptable salts thereof.



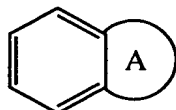
wherein in Formula X:

25

R⁰ represents hydrogen, halogen or C₁₋₆ alkyl;

R¹ represents hydrogen, C₁₋₆ alkyl, C₂₋₆ alkenyl, C₂₋₆ alkynyl, haloC₁₋₆ alkyl, C₃₋₈ cycloalkyl, C₃₋₈ cycloalkylC₁₋₃ alkyl, arylC₁₋₃ alkyl or heteroarylC₁₋₃ alkyl;

R² represents an optionally substituted monocyclic aromatic ring selected from
5 benzene, thiophene, furan and pyridine or an optionally substituted bicyclic ring



attached to the rest of the molecule via one of the benzene ring carbon atoms and
10 wherein the fused ring A is a 5- or 6-membered ring which may be saturated or
partially or fully unsaturated and comprises carbon atoms and optionally one or two
heteroatoms selected from oxygen, sulphur and nitrogen; and

R³ represents hydrogen or C₁₋₃ alkyl, or R¹ and R³ together represent a 3- or 4-
15 membered alkyl or alkenyl chain; and pharmaceutically acceptable salts and solvates
(e.g. hydrates) thereof.

Additional suitable individual compounds of the invention for use in the
treatment include:

20

Cis-2,3,6,7,12,12a-hexahydro-2-(4-pyridylmethyl)-6-(3,4-methylenedioxyphenyl)-
pyrazino[2',1':6,1]pyrido[3,4-b]indole-1,4-dione;

Cis-2,3,6,7,12,12-hexahydro-6-(2,3-dihydrobenzo[b]furan-5-yl)-2-methyl-
25 pyrazino[2',1':6,1]pyrido[3,4-b]indole-1,4-dione;

Cis-2,3,6,7,12,12a-hexahydro-6-(5-bromo-2-thienyl)-2-methyl-
pyrazino[2',1':6,1]pyrido[3,4-b]indole-1,4-dione;

Cis-2,3,6,7,12,12a-hexahydro-2-butyl-6-(4-methylphenyl)-pyrazino[2',1':6,1]pyrido[3,4-b]indole-1,4-dione;

(6R,12aR)-2,3,6,7,12,12a-Hexahydro-2-isopropyl-6-(3,4-methylenedioxyphenyl)-
5 pyrazino[2',1':6,1]pyrido[3,4-b]indole-1,4-dione;

(6R,12aR)-2,3,6,7,12,12a-Hexahydro-2-cyclopentyl-6-(3,4-methylenedioxyphenyl)-
pyrazino[2',1':6,1]pyrido[3,4-b]indole-1,4-dione;

10 (6R,12aR)-2,3,6,7,12,12a-Hexahydro-2-cyclopropylmethyl-6-(4-methoxyphenyl)-
pyrazino[2',1':6,1]pyrido[3,4-b]indole-1,4-dione;

(6R,12aR)-2,3,6,7,12,12a-Hexahydro-6-(3-chloro-4-methoxyphenyl)-2-methyl-
pyrazino[2',1':6,1]pyrido[3,4-b]indole-1,4-dione;

15 (6R,12aR)-2,3,6,7,12,12a-Hexahydro-2-methyl-6-(3,4-methylenedioxyphenyl)-
pyrazino[2',1':6,1]pyrido[3,4-b]indole-1,4-dione;

(6R,12aR)-2,3,6,7,12,12a-Hexahydro-6-(3,4-methylenedioxyphenyl)-
20 pyrazino[2',1':6,1]pyrido[3,4-b]indole-1,4-dione;

(5aR,12R,14aS)-1,2,3,5,6,11,12,14a-Octahydro-12-(3,4-methylenedioxyphenyl)-
pyrrolo[1'',2'':4',5']pyrazino[2',1':6,1]pyrido[3,4-b]indole-5-1,4-dione;

25 Cis-2,3,6,7,12,12a-hexahydro-2-cyclopropyl-6-(3,4-methylenedioxyphenyl)-
pyrazino[2',1':6,1]pyrido[3,4-b]indole-1,4-dione;

(3S,6R,12aR)-2,3,6,7,12,12a-hexahydro-3-methyl-6-(3,4-methylenedioxyphenyl)-
pyrazino[2',1':6,1]pyrido[3,4-b]indole-1,4-dione;

30

and physiologically acceptable salts and solvates (e.g. hydrates) thereof.

Specifically suitable compounds for use in the methods of the invention include:

5 (6R,12aR)-2,3,6,7,12,12a-hexahydro-2-methyl-6-(3,4-methylenedioxyphenyl)-pyrazino[2',1':6,1]pyrido[3,4-b]indole-1,4-dione(Compound A); and

(3S,6R,12aR)-2,3,6,7,12,12a-hexahydro-2,3-dimethyl-6-(3,4-methylenedioxyphenyl)-pyrazino[2',1':6,1]pyrido[3,4-b]indole-1,4-dione (Compound B);

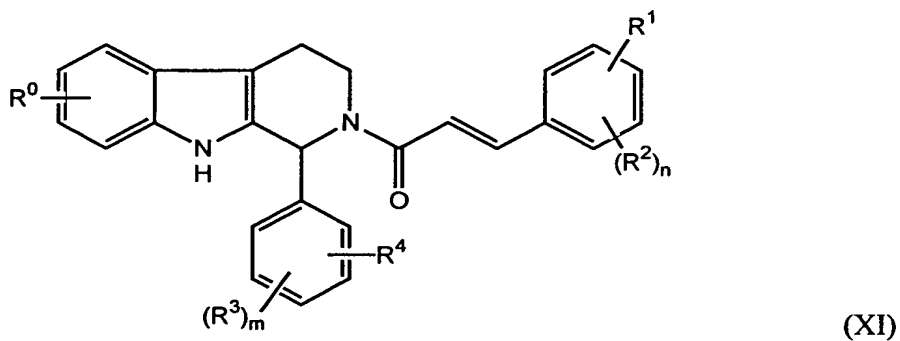
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and physiologically acceptable salts and solvates (e.g. hydrates) thereof.

In another invention embodiment, at least one of the administered compounds is a carboline derivative or N-cinnamoyl derivative or (β) carbolines as described in
15 the U.S. Pat. Nos. 6,043,252 and 6,117,881. Such preferred compounds are set forth in the following Formulae XI and XIII including pharmaceutically acceptable salts thereof.

Compounds of Formula XI are represented by the following structure:

20



wherein in that Formula XI:

25 R⁰ represents hydrogen or halogen;

R¹ is selected from the group consisting of:

- hydrogen, NO₂, trifluoromethyl, trifluoromethoxy, halogen, cyano, a 5- or 6-
membered heterocyclic group containing at least one heteroatom selected from
5 oxygen, nitrogen and sulphur, and is optionally substituted by -C(=O)OR^a or C₁₋₄
alkyl, C₁₋₆ alkyl optionally substituted by -OR^a, C₁₋₃ alkoxy, C(=O)R^a, O-C(=O)R^a,
C(=O)OR^a, C₁₋₄ alkyleneC(=O)OR^a, O-C₁₋₄ alkylene-C(=O)OR^a, C₁₋₄ alkylene-O-C₁₋₄
alkylene-C(=O)OR^a, C(=O)NR^a SO₂ R^c, C(=O)C₁₋₄ alkyleneHet, C₁₋₄ alkyleneNR^a R^b,
C₂₋₆ alkenyleneNR^a R^b, C(=O)NR^a R^b, C(=O)NR^a R^c, C(=O)NR^a C₁₋₄ alkyleneOR^b
10 C(=O)NR^a C₁₋₄ alkyleneHet, OR^a OC₂₋₄ alkylene NR^a R^b, OC₁₋₄ alkylene-CH(OR^a)CH₂
NR^a R^b, O-C₁₋₄ alkylene Het, O-C₂₋₄ alkylene-OR^a, O-C₂₋₄ alkylene-NR^a -C(=O)OR^b,
NR^a R^b, NR^a C₁₋₄ alkyleneNR^a R^b, NR^a C(=O)R^b, NR^a C(=O)NR^a R^b, N(SO₂ C₁₋₄
alkyl)₂, NR^a (SO₂ C₁₋₄ alkyl), SO₂ NR^a R^b, and OSO₂ trifluoromethyl; R² is selected
from the group consisting of: hydrogen, halogen, OR^a, C₁₋₆ alkyl, NO₂, and NR^a R^b,

- 15 or R¹ and R² are taken together to form a 3- or 4-membered alkylene or alkenylene
chain component of a 5- or 6-membered ring, optionally containing at least one
heteroatom;

- 20 R³ is selected from the group consisting of: hydrogen, halogen, NO₂,
trifluoromethoxy, C₁₋₆ alkyl, and C(=O)OR^a ; R⁴ is hydrogen,

- or R³ and R⁴ are taken together to form a 3- or 4- membered alkylene or alkenylene
chain component of a 5- or 6-membered ring, optionally containing at least one
25 heteroatom;

Het represents a 5- or 6-membered heterocyclic group containing at least one
heteroatom selected from the group consisting of oxygen, nitrogen, and sulfur, and is
optionally substituted with C₁₋₄ alkyl;

R^a and R^b can be the same or different, and are independently selected from hydrogen and C₁₋₆ alkyl;

5 R^c represents phenyl or C₄₋₆ cycloalkyl, wherein the phenyl or C₄₋₆ cycloalkyl can be optionally substituted by one or more halogen atoms, one or more -C(=O)OR^a, or one or more -OR^a;

n is an integer selected from 1, 2 and 3;

10 m is an integer selected from 1 and 2;

and pharmaceutically acceptable salts and solvates (e.g., hydrates) thereof.

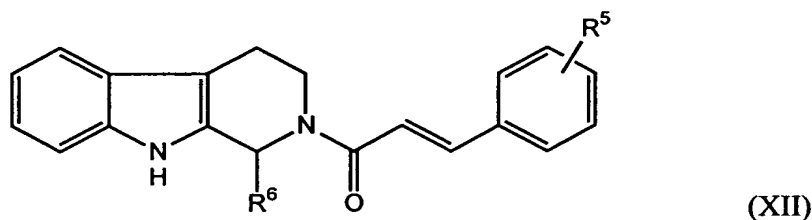
In the above Formula XI, the term alkyl or alkylene as used herein respectively
15 contains the indicated number of carbon atoms and includes straight chained and branched alkyl or alkylene groups, typically methyl, methylene, ethyl, and ethylene groups, and straight chained and branched propyl, propylene, butyl, and butylene groups. The term C₂₋₆ alkenylene as used with respect to Formula XI means groups that contain 2 to 6 carbon atoms and includes straight chained and branched
20 alkenylene groups, in particular ethenylene or the like. In Formula XI, the term C₄₋₆ cycloalkyl denotes cyclic groups containing 4 to 6 carbon atoms, namely cyclobutane, cyclopentane, and cyclohexane. In Formula XI, the term halogen as used herein includes fluorine, chlorine, bromine, and iodine. In Formula XI, the term 5- or 6-membered heterocyclic group as used herein includes 5- or 6- membered
25 heterocycloalkyl and heteroaryl groups, e.g., tetrahydrofuranyl, piperidyl, piperazinyl, pyrrolidinyl, morpholinyl, pyridyl, imidazolyl, furyl, and tetrazolyl. In Formula XI, appropriately, R⁰ represents hydrogen. Alternatively, R⁰ can represent halogen, in particular fluorine. In Formula XI, R¹ may suitably represent any of -OR^a, -O-C₂₋₄ alkyleneNR^a R^b, -O-C₁₋₄ alkyleneHet and -O-C₂₋₄ alkylene-OR^a. In particular, R¹
30 represents -O-C₂₋₄ alkyleneNR^a R^b, wherein C₂₋₄ alkylene can represent ethylene, and, R^a and R^b can independently represent methyl. Particularly suitably R² represents

hydrogen. Alternatively, in the case where R^1 and R^2 together form a 3- or 4-membered alkylene or alkenylene chain component of a 5- or 6-membered ring, optionally containing at least one heteroatom as hereinbefore described, R^1 and R^2 together form a methylenedioxy chain, an ethyleneoxy chain, an ethylenedioxy chain, an ethenyleneoxy chain, a propylene chain, a butylene chain or $-NR^a$ ethylene-O- .
 5 Aptly, R^1 and R^2 together form methylenedioxy, propylene, or $-N(CH_3)-(CH_2)_2-O-$.

In the above Formula XI, suitably R^3 and R^4 taken together form a 3- or 4-membered alkylene or alkenylene chain component of a 5- or 6-membered ring, optionally containing at least one heteroatom as hereinbefore described. Particularly
 10 R^3 and R^4 together form a methylenedioxy chain, an ethyleneoxy chain, an ethylenedioxy chain, an ethenyleneoxy chain, a propylene chain, a butylene chain, or $-NR^a$ ethylene-O-. Aptly R^3 and R^4 together form a methylenedioxy chain, an ethyleneoxy chain, an ethylenedioxy chain, an ethenyleneoxy chain, or a propylene
 15 chain. In particular, R^3 and R^4 together form methylenedioxy or ethyleneoxy, most particularly ethyleneoxy.

A particular subgroup of compounds for use in the methods of the invention include those of the following formula (XII)

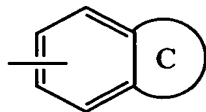
20



wherein

25 R^5 is selected from the group consisting of $-OH$, $-OC_{2-4}$ alkylene NR^a R^b , and $O-C_{1-4}$ alkylene Het, wherein Het is as hereinbefore described, and

R⁶ represents



- 5 wherein C represents a 5- or 6-membered ring which can be saturated or partially or fully unsaturated, and comprises carbon atoms and optionally one or two heteroatoms selected from oxygen, sulphur and nitrogen, optionally substituted by C₁₋₄ alkyl;

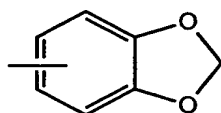
and pharmaceutically acceptable salts and solvates (e.g., hydrates thereof).

10

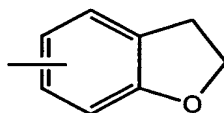
In that Formula XII, typically, R⁵ represents -OC₂₋₄ alkylene NR^aR^b, in particular -OCH₂CH₂N(CH₃)₂. Alternatively, R⁵ can represent -O-C₁₋₄ alkylene Het, where Het can be piperidyl, pyrrolidinyl (optionally substituted by C₁₋₄ alkyl, e.g., methyl) or morpholinyl.

15

Particularly R⁶ represents

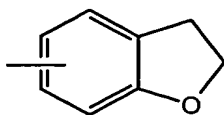


20 or



especially

25



Additional particular compounds for use in the methods of the invention include:

5 (E)-1-(1-Phenyl-1,3,4,9-tetrahydro-β-carboline-2-yl)-3-phenylpropene-1-one;

(E)-1-(1-Phenyl-1,3,4,9-tetrahydro-β-carboline-2-yl)-3-(4-nitrophenyl)propene-1-one;

(E)-1-(1-Phenyl-1,3,4,9-tetrahydro-β-carboline-2-yl)-3-(4-trifluoromethylphenyl)propene-1-one;

10

(E)-1-(1-Phenyl-1,3,4,9-tetrahydro-β-carboline-2-yl)-3-(4-methoxyphenyl)propene-1-one;

(E)-1-[1-(4-Methoxyphenyl)-1,3,4,9-tetrahydro-β-carboline-2-yl]-3-(4-trifluoromethylphenyl) propene-1-one;

15

(E)-N-[4-[3-Oxo-3-(1-phenyl-1,3,4,9-tetrahydro-β-carboline-2-yl)propenyl]phenyl]acetamide;

20 (E)-1-[1-(4-Methoxyphenyl)-1,3,4,9-tetrahydro-β-carboline-2-yl]-3-phenylpropene-1-one;

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro-β-carboline-2-yl]-3-phenylpropene-1-one;

25

(E)-1-(1-Phenyl-1,3,4,9-tetrahydro-β-carboline-2-yl)-3-(4-formylphenyl) propene-1-one;

(E)-N-[4-[3-Oxo-3-(1-(4-nitrophenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl)propenyl]phenyl]acetamide;

(E)-1-[1-(4-Nitrophenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-phenylpropene-1-one;

5

(E)-1-[1-(4-Trifluoromethoxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-phenylpropene-1-one;

(E)-1-[1-(4-Methylphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-phenylpropene-1-one;

10

(E)-N-[4-[3-Oxo-3-(1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl)-propenyl]phenyl]acetamide;

(E)-4-[3-Oxo-3-(1-phenyl-1,3,4,9-tetrahydro- β -carbolin-2-yl)-propenyl]benzoic acid, methyl ester;

15

(E)-1-[1-(2-Chlorophenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-phenylpropene-1-one;

20

(E)-1-(1-Phenyl-1,3,4,9-tetrahydro- β -carbolin-2-yl)-3-(3,4-methylenedioxyphenyl)-propene-1-one;

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-bromophenyl)-propene-1-one;

25

(E)-1-[1-(4-Chlorophenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-phenylpropene-1-one;

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-ethoxyphenyl)propene-1-one;

30

(E)-4-[3-Oxo-3-(1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl)propenyl]acetic acid, phenyl ester;

- 5 (E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-hydroxyphenyl)propene-1-one;

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-formylphenyl)propene-1-one;

10

(E)-1-[4-[3-Oxo-3-(1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl)-propenyl]phenyl]-3-phenylurea;

15

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-aminophenyl)propene-1-one;

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-nitrophenyl)propene-1-one;

20

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-[(4-bis(methylsulfonyl)-aminophenyl)-propene-1-one];

(E)-4-[3-Oxo-3-[1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-propenyl]benzoic acid, methyl ester;

25

(E)-N-[4-[3-Oxo-3-[1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]propenyl]phenyl]methanesulfonamide;

30

(E)-4-[3-Oxo-3-[1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]propenyl]benzamide];

(E)-4-[3-Oxo-3-[1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-propenyl]benzoic acid;

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-cyanophenyl)propene-1-one;

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-trifluoromethylphenyl)propene-1-one;

10 (E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(3,4-methylenedioxyphenyl)propene-1-one;

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-chlorophenyl)-propene-1-one;

15 (E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-trifluoromethoxyphenyl)propene-1-one;

20 (E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-methylphenyl)propene-1-one;

(E)-[4-[3-Oxo-3-(1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl)propenyl]phenyl]urea;

25 (E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-hydroxymethylphenyl)propene-1-one;

(E)-N-Benzyl-4-[3-oxo-3-(1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro-beta.-carbolin-2-yl)propenyl]benzamide;

30

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(2,4-dichlorophenyl)propene-1-one;

5 (E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(3-methoxy-4-hydroxyphenyl)propene-1-one;

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(3-hydroxy-4-methoxyphenyl)propene-1-one;

10 (E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-fluorophenyl)-propene-1-one;

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-indan-5-yl-1-propene-1-one;

15 (E)-N-[4-[3-Oxo-3-(1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl)propenyl]benzoyl]benzenesulfonamide;

20 (E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(3,4-dichlorophenyl)propene-1-one;

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(3,4-dimethoxyphenol)propene-1-one;

25 (E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(3,4-dihydroxyphenyl)propene-1-one;

(E)-N-Methyl-N-[4-[3-oxo-3-(1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl)propenyl]phenyl]acetamide;

30

(E)-2,2-Dimethyl-N-[4-[3-Oxo-3-(1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro-
β-carbolin-2-yl)propenyl]phenyl]propionamide;

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro-β-carbolin-2-yl]-3-(3,5-
5 dimethoxyphenyl)propene-1-one;

(E)-(N)-{4-[3-[1-(3,4-Methylenedioxyphenyl)-6-fluoro-1,3,4,9-tetrahydro-beta-
carbolin-2-yl]-3-oxopropenyl]-phenyl}-acetamide;

10 (E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro-β-carbolin-2-yl]-3-(3,4,5-
trimethoxyphenyl)propene-1-one;

(E)-N-[4-[3-Oxo-3-(1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro-β-carbolin-2-
yl)propenyl]phenyl]isobutyramide;

15 (E)-1-[1-(3,4-Methylenedioxyphenyl)-6-fluoro-1,3,4,9-tetrahydro-β-carbolin-2-yl]-3-
phenylpropene-1-one;

(E)-N-(2-Methoxyethyl)-4-[3-oxo-3-(1-(3,4-methylenedioxyphenyl)-1,3,4,9-tet
20 rahydro-β-carbolin-2-yl)propenyl]benzamide;

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro-β-carbolin-2-yl]-3-(3-
hydroxyphenyl)propene-1-one;

25 (E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro-β-carbolin-2-yl]-3-(3-
methoxyphenyl)propene-1-one;

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro-β-carbolin-2-yl]-3-(3-
nitrophenyl)propene-1-one;

30

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-[4-(2-dimethylaminoethoxy)phenyl]propene-1-one;

(E)-N-(2-Morpholin-4-ylethyl)-4-[3-oxo-3-(1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl)propenyl]benzamide;

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-[4-(1H-tetrazol-5-yl)phenyl]propene-1-one;

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(3-aminophenyl)propene-1-one;

(E)-N-Cyclohexyl-4-[3-oxo-3-(1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl)propenyl]benzamide;

(E)-N-(Tetrahydrofuran-2-ylmethyl)-4-[3-oxo-3-(1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl)propenyl]benzamide;

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(3-cyanophenyl)propene-1-one;

(E)-N-(4-Piperidine-4-carboxylic acid, ethyl ester)-4-[3-oxo-3-(1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl)propenyl]benzamide;

(E)-N-(4-Piperidine-4-carboxylic acid)-4-[3-oxo-3-(1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl)propenyl]benzamide;

(E)-3-[3-Oxo-3-[1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]propenyl]benzoic acid

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(3-(4-methylpiperazine-1-carbonyl)-phenyl)propene-1-one

5 (E)-N-(2-Piperazin-1-ylethyl)-3-[3-oxo-3-(1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl)propenyl]benzamide

(E)-4-[3-Oxo-3-(1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl)-propenyl]acetic acid ethyl ester

10 (E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(3-tetrazolophenyl)propene-1-one

(E)-2-[3-Oxo-3-[1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-propenyl]benzoic acid, methyl ester

15

(E)-3-[3-Oxo-3-[1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-propenyl]benzoic acid, methyl ester

20 (E)-1-(4-[3-Oxo-3-(1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl)-propenyl]phenyl)piperidine-4-carboxylic acid, ethyl ester

(E)-N-(1-Ethylpyrrolidin-2-yl-methyl)-3-[3-oxo-3-(1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl)propenyl]benzamide

25 (E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(3-(2-dimethylaminoethoxy)phenyl)propene-1-one

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(3,5-diterbutyl-4-hydroxyphenyl)propene-1-one

30

(E)-3-[3-Oxo-3-[1-(4-methoxycarbonylphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]propenyl]benzoic acid, methyl ester

5 (E)-2-[3-Oxo-3-[1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]propenyl]benzoic acid

(E)-(4-[3-Oxo-3-(1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl)propenyl]phenoxy)acetic acid, ethyl ester

10 (E)-(4-[3-Oxo-3-(1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl)propenyl]phenyl)acetic acid

(E)-(4-[3-Oxo-3-(1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl)propenyl]phenoxy)acetic acid

15

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(3-nitro-4-chlorophenyl)propene-1-one

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(5-nitro-2-chlorophenyl)propene-1-one

20

(E)-3-Chloro-4-[3-oxo-3-[1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]propenyl]benzoic acid, methyl ester

25 (E)-(4-[3-Oxo-3-(1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl)propenyl]benzyloxy)acetic acid

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(5-amino-2-chlorophenyl)propene-1-one

30

(E)-3-Chloro-4-[3-oxo-3-[1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro-beta-carbolin-2-yl]propenyl]benzoic acid

5 (E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro-β-carbolin-2-yl]-3-(3,5-dibromo-4-hydroxyphenyl)propene-1-one

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro-β-carbolin-2-yl]-3-(4-(2-dimethylaminopropoxy)phenyl)propene-1-one

10 (E)-2-Chloro-5-[3-oxo-3-[1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro-beta-carbolin-2-yl]propenyl]benzoic acid, methyl ester

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro-β-carbolin-2-yl]-3-(4-(2-diisopropylaminoethoxy)phenyl)propene-1-one

15 (E)-2-Chloro-5-[3-oxo-3-[1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro-beta-carbolin-2-yl]propenyl]benzoic acid

20 (E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro-β-carbolin-2-yl]-3-(3-hydroxy-4-nitro-phenyl)propene-1-one

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro-β-carbolin-2-yl]-3-(3,5-dimethyl-4-hydroxyphenyl)propene-1-one

25 (E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro-β-carbolin-2-yl]-3-(3-(2-dimethylaminoethoxy)-4-nitro-phenyl)propene-1-one

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro-β-carbolin-2-yl]-3-(3-(2-dimethylaminoethoxy)-4-amino-phenyl)propene-1-one

30

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(3-nitro-4-hydroxy-5-methoxyphenyl)propene-1-one

5 (E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(3-chlorophenyl)propene-1-one

(E)-1-[1-(4-Methoxy-phenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(2-chloro-5-nitrophenyl)propene-1-one

10 (E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(2,6-dichlorophenyl)propene-1-one

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-methylaminomethylphenyl)propene-1-one

15 (E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(3-methylphenyl)propene-1-one

20 (E)-N-Methyl-(4-[3-oxo-3-(1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro-beta-carbolin-2-yl)propenyl]benzenesulfonamide

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(3-hydroxy-4-acetylphenyl)propene-1-one

25 (E)-1-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(2-chloro-5-nitrophenyl)propene-1-one

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(2-hydroxyphenyl)propene-1-one

30

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(3-nitro-2-piperidin-1-ylphenyl)propene-1-one

5 (E)-1-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-phenylpropene-1-one

(E)-1-[1-(4-Isopropylphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(3-nitrophenyl)propene-1-one

10 (E)-1-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(3-nitrophenyl)propene-1-one

(E)-(R)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-phenylpropene-1-one

15 (E)-(S)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-phenylpropene-1-one

20 (E)-1-[1-(4-Methoxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(3-nitrophenyl)propene-1-one

(E)-1-[1-(4-Methylphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(2-chloro-5-nitrophenyl)propene-1-one

25 (E)-N-(Tetrahydrofuran-2-ylmethyl)-3-[3-oxo-3-(1-(3,4-methylenedioxy)-1,3,4,9-tetrahydro- β -carbolin-2-yl)propenyl]benzamide

(E)-1-[1-(Indan-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-phenylpropene-1-one

30 (E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(3-acetylphenyl)propene-1-one

(E)-1-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-(2-dimethylaminoethoxy)phenyl)propene-1-one

- 5 (E)-4-[3-Oxo-3-[1-(4-methoxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]propenyl]benzoic acid, methyl ester

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-methyl-3,4-dihydro-2H-benzo[1,4]-oxazin-6-yl)propene-1-one

10

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(2-hydroxy-5-nitrophenyl)propene-1-one

- 15 (E)-4-[3-Oxo-3-[1-(2,3-dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]propenyl]benzoic acid, methyl ester

(E)-4-[3-Oxo-3-[1-(4-methoxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]propenyl]benzoic acid

- 20 (E)-4-[3-Oxo-3-[1-(2,3-dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]propenyl]benzoic acid

(E)-1-[1-(Benzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-phenylpropene-1-one

25

(E)-3-[3-Oxo-3-(1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl)-propenyl]phenyltrifluoromethanesulfonic acid, phenyl ester

- 30 (E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-[4-(2-hydroxyethoxy)phenyl]propene-1-one

(E)-1-[1-(Benzofuran-5-yl-1,3,4,9-tetrahydro- β -carbolin-2-yl)]-3-(4-(2-dimethylaminoethoxy)phenyl)propene-1-one

5 (E)-1-[1(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl] -3-(2-dimethylaminophenyl)propene-1-one

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(2-piperidin-1-ylphenyl)propene-1-one

10 (E)-4-[3-Oxo-3-[1-(benzofuran-5-yl-1,3,4,9-tetrahydro- β -carbolin-2-yl)]-propenyl]-benzoic acid, methyl ester

(E)-4-[3-(1-Benzofuran-5-yl-1,3,4,9-tetrahydro- β -carbolin-2-yl)-3-oxo-propenyl]-benzoic acid

15 (E)-4-[3-Oxo-3-(1-(3,4-methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl)propenyl]phenyl)trifluoromethanesulfonic acid, phenyl ester

20 (E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(2-(2-dimethylaminoethoxy)phenyl)propene-1-one

(E)-1-[1-(3-Fluoro-4-methoxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-phenylpropene-1-one

25 (E)-(R)-1-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl)]-3-(4-(2-dimethylaminoethoxy)phenyl)propene-1-one

(E)-[1-(2,3-Dihydrobenzo[1,4]dioxin-6-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-phenylpropene-1-one

30

(E)-1-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-(2-pyrrolidin-1-ylethoxy)phenyl)propene-1-one

5 (E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-[4-pyrrolidin-1-ylphenyl]propene-1-one

(E)-(R)-1-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(3-nitrophenyl)propene-1-one

10 (E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-[4-imidazol-1-ylphenyl]propene-1-one

(E)-4-[3-[1-(2,3-Dihydrobenzo[1,4]dioxin-6-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-oxo-propenyl]benzoic acid, methyl ester

15 (E)-1-[1-(2,3-Dihydrobenzo[1,4]dioxin-6-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(3-nitrophenyl)propene-1-one

20 (E)-1-[1-(2,3-Dihydrobenzo[1,4]dioxin-6-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-(2-dimethylaminoethoxy)phenyl)propene-1-one

(E)-1-[1-(3-Fluoro-4-methoxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-(2-dimethylaminoethoxy)phenyl)propene-1-one

25 (E)-4-[3-[1-(2,3-Dihydrobenzo[1,4]dioxin-6-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-oxopropenyl]benzoic acid

(E)-(R)-1-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-phenylpropene-1-one

30

(E)-(S)-1-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-(2-dimethylaminoethoxy)phenyl)propene-1-one

5 (E)-1-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-aminophenyl)propene-1-one

(E)-(S)-1-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-phenylpropene-1-one

10 (E)-(S)-1-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(3-nitrophenyl)propene-1-one

(E)-(R)-1-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-(1-(S)-methylpyrrolidin-2-yl-methoxy)phenyl)propene-1-one

15 (E)-(R)-1-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(3-hydroxyphenyl)propene-1-one

20 (E)-(R)-1-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-(2-dimethylamino-1-methylethoxy)phenyl)propene-1-one

(E)-1-(1-Phenyl-1,3,4,9-tetrahydro- β -carbolin-2-yl)-3-(4-(4-methylpyperazin-1-yl)-phenyl)propene-1-one

25 (E)-(R)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-(1-(S)-methylpyrrolidin-2-yl-methoxy)phenyl)propene-1-one

(E)-(R)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-(2-dimethylamino-1-methylethoxy)phenyl)propene-1-one

30

(E)-(R)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl)]-3-(4-(2-dimethylaminopropoxy)phenyl)propene-1-one

5 (E)-4-[3-Oxo-3-[1-(3,4-fluorophenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]propenyl]benzoic acid, methyl ester

(E)-(R)-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl)]-3-(4-(2-diethylaminoethoxy)phenyl)propene-1-one

10 (E)-(R)-1-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl)]-3-(4-(2-dimethylaminopropoxy)phenyl)propene-1-one

(E)-4-[3-Oxo-3-[1-(3,4-difluorophenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]propenyl]benzoic acid

15 (E)-(R)-1-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl)]-3-(4-aminophenyl)propene-1-one

20 (E)-(R)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl)]-3-(4-aminophenyl)propene-1-one

(E)-(R)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl)]-3-(4-(2-pyrrolidin-1-ylethoxy)phenyl)propene-1-one

25 (E)-(R)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl)]-3-(4-(2-diethylaminoethoxy)phenyl)propene-1-one

(E)-1-[1-(3-Fluoro-4-methoxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl)]-3-(3-nitrophenyl)propene-1-one

30

(E)-(R)-1-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-trifluoromethylphenyl)propene-1-one

5 (E)-(R)-1-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(3-trifluoromethylphenyl)propene-1-one

(E)-(R)-1-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-(2-morpholin-4-ylethoxy)phenyl)propene-1-one

10 (E)-(R)-1-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-(2-(ethylmethylamino)ethoxy)phenyl)propene-1-one

(E)-1-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-(3-(dimethylamino)propenyl)phenyl)propene-1-one

15 (E)-(R)-1-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-(3-dimethylamino-2-hydroxypropoxy)phenyl)propene-1-one

20 (E)-(R)-1-(1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl)-3-(4-formylphenyl)propene-1-one

(E)-(R)-1-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-propylaminomethyl)phenyl)propene-1-one

25 (E)-(R)-1-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-[4-(2-dimethylaminoethylamino)phenyl]propene-1-one

(E)-(R)-1-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-(2-aminoethoxy)phenyl)propene-1-one

30

(E)-(R)-1-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-hydroxyphenyl)propene-1-one

(E)-(R)-1-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-
5 (4-methylpiperazin-1-yl)phenyl)propene-1-one

(E)-(R)-1-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-methylaminomethyl)phenyl)propene-1-one

10 (E)-(R)-1-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-isopropylaminomethyl)phenyl)propene-1-one

(E)-(R)-1-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-dimethylaminomethyl)phenyl)propene-1-one

15 (E)-(R)-1-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-[4-(3-dimethylaminopropoxy)phenyl]propene-1-one

(E)-(R)-1-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-
20 (2-piperidin-1-ylethoxy)phenyl)propene-1-one

(E)-1-[1-(3,4-Methylenedioxyphenyl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-(2-piperidin-1-yl-ethoxy)phenyl)propene-1-one

25 (E)-(R)-[2-(4-{3-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-oxopropenyl}-phenoxy)ethyl]methylcarbamic acid, tertbutyl ester

(E)-(R)-1-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-[4-(2-methylaminoethoxy)phenyl]propene-1-one

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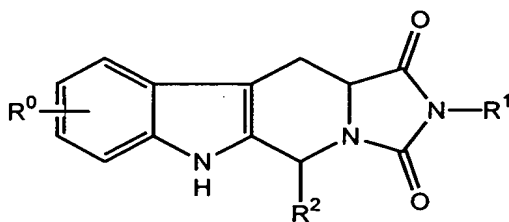
and pharmaceutically acceptable salts and solvates (e.g., hydrates) thereof.

A specific compound of the invention is:

(E)-(R)-1-[1-(2,3-Dihydrobenzofuran-5-yl)-1,3,4,9-tetrahydro- β -carbolin-2-yl]-3-(4-
5 (2-dimethylaminoethoxy)phenyl)propene-1-one,

and pharmaceutically acceptable salts and solvates (e.g., hydrates) thereof.

In another invention embodiment, at least one of the administered delivery
10 compounds or agents is a chemical compound described in the U.S. Pat. Nos.
6,143,757 and 6,001,847. Such preferred compounds are set forth in the following
Formula XIII including pharmaceutically acceptable salts thereof.



15

wherein in Formula XIII:

R⁰ represents hydrogen, halogen, or C₁₋₆ alkyl;

20 R¹ is selected from the group consisting of:

(a) hydrogen,

(b) C₁₋₆ alkyl, optionally substituted with one or more substituents selected from
25 phenyl, halogen, --CO₂R_a and -NR_aR_b,

(c) C₃₋₆ cycloalkyl,

(d) phenyl, and

- 5 (e) a 5- or 6-membered heterocyclic ring containing at least one heteroatom selected from oxygen, nitrogen and sulphur, and being optionally substituted with one or more C_{1-6} alkyl, and optionally linked to the nitrogen atom to which R_1 is attached via C_{1-6} alkyl;

R_2 is selected from the group consisting of:

10

(f) C_{3-6} cycloalkyl,

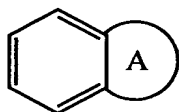
(g) phenyl, optionally substituted with one or more substituents selected from $-OR_a$, $-NR_aR_b$, halogen, hydroxy, trifluoromethyl, cyano, and nitro,

15

(h) a 5- or 6-membered heterocyclic ring containing at least one heteroatom selected from oxygen, nitrogen and sulphur; and

(i) a bicyclic ring

20



attached to the rest of the molecule via one of the benzene ring carbon atoms, wherein A is a 5- or 6-membered heterocyclic ring as defined in (h); and

25

R_a and R_b , independently, represent hydrogen or C_{1-6} alkyl.

In the above Formula XIII, the term " C_{1-6} alkyl" denotes any straight or branched alkyl chain containing 1 to 6 carbon atoms, and includes methyl, ethyl, n-

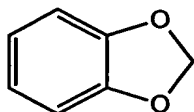
propyl, iso-propyl, n-butyl, pentyl, hexyl, and the like. The term "halogen" denotes fluorine, chlorine, bromine, and iodine.

A particular group of compounds according to Formula XIII are those wherein
5 R_0 represents any of hydrogen, methyl, bromine, and fluorine, but the definition of R_0 given in Formula XIII includes within its scope other C_{1-6} -alkyl and halogen groups.

In Formula XIII above, R_1 preferably can represent a substituent selected from methyl, ethyl (optionally substituted by one or more chlorine atoms), butyl,
10 cyclohexyl and benzyl. Other R_1 substituents include hydrogen; cycloalkyl groups, such as cyclopropyl; C_{1-6} alkyl, typically ethyl or propyl, substituted by an $-NR_aR_b$ substituent, such as a dimethylamino substituent; phenyl optionally linked to the nitrogen atom to which R_1 is attached via a C_{1-6} alkyl chain, such as ethyl or the like; and C_{1-6} alkyl, e.g., methyl, substituted by $-CO_2 R_a$, such as $-CH_2CO_2$ Et (Et is
15 CH_2CH_3) and the like.

Suitable heterocyclic rings within the definition of R_1 of Formula XIII include pyridyl, morpholinyl, piperazinyl, pyrrolidinyl, and piperidinyl. Generally, such heterocyclic rings are linked to the nitrogen atom to which R_1 is attached via a C_{1-6}
20 alkyl chain, more appropriately a C_{1-4} alkyl chain.

A particular substituent represented by R_2 is



25

Other R_2 substituents include thienyl, pyridyl, furyl, and phenyl, wherein phenyl can be substituted with one or more substituents selected from $-OR_a$ (e.g., methoxy), $-NR_aR_b$ (e.g., dimethylamino), halogen (in particular chlorine or fluorine),

hydroxy, trifluoromethyl, cyano, and nitro. Alternatively, R_2 can represent a C_{3-6} cycloalkyl group, such as cyclohexyl or the like.

The pharmaceutically acceptable salts of the compounds of Formula XIII that
5 contain a basic center are acid addition salts formed with pharmaceutically acceptable acids. Examples include the hydrochloride, hydrobromide, sulfate or bisulfate, phosphate or hydrogen phosphate, acetate, benzoate, succinate, fumarate, maleate, lactate, citrate, tartrate, gluconate, methanesulphonate, benzenesulphonate, and p-toluenesulphonate salts. Compounds of Formula XIII also can provide
10 pharmaceutically acceptable metal salts, in particular alkali metal salts, with bases. Examples include the sodium and potassium salts.

It is to be understood that the present invention covers all appropriate combinations of particular and preferred groupings hereinabove.

15

Additional preferred PDE inhibitor compounds for use in accordance with the invention may be identified by simple testing, such as in by exhibiting an ID50 of less than about 10 mM, preferably less than about 1mM in an in vitro assay for determining PDE or PDE-5 inhibitory action as disclosed in U.S. Patent 6,100,270;
20 WO-A-93/06104; vWO-A-93/07149; WO-A-93/12095; and WO-A-94/00453.

As discussed above, additional permeability agents distinct from a PDE inhibitor can be employed in accordance with the invention. The suitability of any specific additional permeability agent can be readily evaluated by testing the agent to
25 determine if the candidate agent enhances uptake of nucleic acid by targeted tissue relative to a control tissue sample that has not been exposed to the candidate permeability agent. For example, a candidate permeability suitably may provide at least about 10, 20, 30, 40, 50, 60, 70, 80 or 90 percent greater uptake of nucleic acid by targeted tissue relative to a control tissue sample that has not been exposed to the
30 candidate permeability agent. In certain applications, use of a permeability agent having even greater uptake of nucleic acid may be preferred, e.g. where the

permeability agent provides at least about 100, 125, 150, 175, 200, 300, 400, 500, 600, 800 or 1000 percent greater uptake of nucleic acid by targeted tissue relative to a control tissue sample that has not been exposed to the permeability agent. It also should be appreciated that the level of nucleic agent uptake that will be suitable for various applications can differ. Thus, for certain applications, lower levels of nucleic acid uptake (e.g. nucleic acid uptake in 2% of cells in a target organ or mass of cells) may be suitable for one application, whereas for other applications, higher levels of nucleic acid uptake (e.g. nucleic acid uptake approaching 90 or 100% of cells in a target organ or mass of cells) may be optimal. The conditions herein described enable graded levels of nucleic acid uptake, e.g. by appropriate varying of type and quantity of permeability agent, virus amount or concentration, and virus exposure time.

Suitable permeability agents and amounts thereof (including PDE inhibitor compounds as well as additional, distinct permeability agents such as serotonin) generally will exhibit good activity in a standard *in vitro* vasculature permeability assay. For example, permeability agents preferred for at least many applications will include those compounds that exhibit at least about 5%, more preferably at least about 10%, still more preferably at least about 25% or 50% of the permeability activity exhibited by an equivalent molar concentration of bradykinin in a standard vasculature permeability assay, specifically the assay disclosed in R.J. Mullins et al., *Journal of Trauma* 1989, 29(6):1053-1063. That disclosed assay includes measuring skin lymph flow (LYM FLOW $\mu\text{l}/\text{min}$) after perfusing a solution containing the permeability agent through specified vasculature of a test animal for a specified period of time. References herein to a standard permeability assay are intended to refer to that protocol and as disclosed in R.J. Mullins, *supra*.

Specifically suitable amounts of a particular phosphodiesterase inhibitor (PDE) compound(s) and optional distinct permeability agent is exemplified in the example which follows and can be determined by simple testing, e.g. conducting assays as shown in the example which follows to determine at what concentration of a

particular PDE inhibitor compound and optional distinct agent are optimal gene transfer realized.

It also should be appreciated that optimal permeability agents and conditions
5 for a particular application may vary with a number of factors such as the specific
nucleic acid being administered, the solid cell mass that is being treated and the like.
Thus, while histamine may be suitable for many applications of the invention,
histamine may be less suitable for certain applications and is excluded from certain
aspects of the invention. Again, the suitability of any particular agent can be readily
10 determined by simple testing as discussed above.

Nucleic acid administered in accordance with the invention may be any
nucleic acid (DNA or RNA) including genomic DNA, cDNA, rRNA, mRNA and
tRNA. These constructs may encode a gene product of interest, e.g. a therapeutic or
15 diagnostic agent. A wide variety of known polypeptides are known that may be
suitably administered to a patient in accordance with the invention.

For instance, for administration to cardiac myocytes, nucleic acids that encode
vasoactive factors may be employed to treat vasoconstriction or vasospasm. Nucleic
20 acids that encode angiogenic growth factors may be employed to promote
revascularization. Suitable angiogenic growth factors include e.g. the fibroblast
growth factor (FGF) family, endothelial cell growth factor (ECGF) and vascular
endothelial growth factor (VEGF; see U.S. Patents 5,332,671 and 5,219,739). See
Yanagisawa-Miwa et al., *Science* 1992, 257:1401-1403; Pu et al., *J Surg Res* 1993,
25 54:575-83; and Takeshita et al., *Circulation* 1994, 90:228-234. Additional agents that
may be administered to ischemic heart conditions, or other ischemic organs include
e.g. nucleic acids encoding transforming growth factor α (TGF- α), transforming
growth factor β (TGF- β), tumor necrosis factor α and tumor necrosis factor β .
Suitable vasoactive factors that can be administered in accordance with the invention
30 include e.g. atrial natriuretic factor, platelet-derived growth factor, endothelin and the
like. Additional agents that may be administered for the prevention of atrial or

ventricular arrhythmias include ion channel proteins and/or functionally effective subunits and/or fragments of ion channel proteins that can function to either overexpress or inhibit certain ion currents in the hearts. Further regulatory sequences, such as promoter elements may be added to restrict expression to specific regions of an organ, such as the heart (e.g., atrial natriuretic factor promoter produces preferential expression in the atria relative to the ventricles). Alternatively, regulatory sequences may be added so that expression is regulated up or down by an administered or endogenous compound, such as a hormone.

10 For treatment of malignancies, particularly solid tumors, nucleic acids encoding various anticancer agents can be employed, such as nucleic acids that code for diphtheria toxin, thymidine kinase, pertussis toxin, cholera toxin and the like. Nucleic acids encoding antiangiogenic agents such as matrix metalloproteases and the like also can be employed. See J.M. Ray et al. *Eur Respir J* 1994, 7:2062-2072.

15 For other therapeutic applications, polypeptides transcribed by the administered nucleic acid can include growth factors or other regulatory proteins, a membrane receptor, a structural protein, an enzyme, a hormone and the like.

20 Also, as mentioned above, the invention provides for inhibiting expression or function of an endogenous gene of a subject. This can be accomplished by several alternative approaches. For example, antisense nucleic acid may be administered to a subject in accordance with the invention. Typically, such antisense nucleic acids will be complementary to the mRNA of the targeted endogenous gene to be suppressed, or
25 to the nucleic acid that codes for the reverse complement of the endogenous gene. See J.H. Izant et al., *Science* 1985, 229:345-352; and L.J. Maher II et al., *Arch Biochem Biophys* 1987, 253:214-220. Antisense modulation of expression of a targeted endogenous gene can include antisense nucleic acid operably linked to gene regulatory sequences.

30

Alternatively, nucleic acid may be administered which antagonizes the expression of selected endogenous genes (e.g. ribozymes), or otherwise interferes with function of the endogenous gene or gene product.

5 The nucleic acid to be administered can be obtained by known methods, e.g. by isolating the nucleic acids from natural sources or by known synthetic methods such as the phosphate triester method. See, for example, *Oligonucleotide Synthesis*, IRL Press (M.J. Gait, ed. 1984). Synthetic oligonucleotides also may be prepared using commercially available automated oligonucleotide synthesizers. Also, as is
10 known, if the nucleic acid to be administered is mRNA, it can be readily prepared from the corresponding DNA, e.g. utilizing phage RNA polymerases T3, T7 or SP6 to prepare mRNA from the DNA in the presence of ribonucleoside triphosphates. The nucleotide sequence of numerous therapeutic and diagnostic peptides including those discussed above are disclosed in the literature and computer databases (e.g., GenBank,
15 EMBL and Swiss-Prot). Based on such information, a DNA segment may be chemically synthesized or may be obtained by other known routine procedures such as PCR.

 To facilitate manipulation and handling of the nucleic acid to be administered,
20 the nucleic acid is preferably inserted into a cassette where it is operably linked to a promoter. The promoter should be capable of driving expression in the desired cells. The selection of appropriate promoters can be readily accomplished. For some applications, a high expression promoter is preferred such as the 763-base pair cytomegalovirus (CMV) promoter. The Rous sarcoma (RSV) (Davis et al., *Hum*
25 *Gene Ther*, 1993, 4:151) and MMT promoters also may be suitable. Additionally, certain proteins can be expressed using their native promoter. Promoters that are specific for selected cells also may be employed to limit transcription in desired cells. Other elements that can enhance expression also can be included such as an enhancer or a system that results in high expression levels such as a *tat* gene or a *tar* element. A
30 cloning vehicle also may be designed with selective receptor binding and using the promoter to provide temporal or situational control of expression.

The cassette then can be inserted into a vector, e.g. a viral vector such as adenoviruses, adeno-associated virus, retroviruses, herpes viruses (e.g. herpes simplex virus), vaccinia viruses, papoviruses, the Sendai virus, the SV40 virus, hybrid viruses, and the like. As mentioned above, adenoviruses are generally preferred. Lytic and semi-lytic viral vectors also can be employed, particularly for administration to malignant cells, such as cells of a solid tumor. Replication-defective recombinant adenoviral vectors, and other suitable vectors, can be produced by known procedures. See Quantin et al., *Proc Natl Acad Sci USA* 1992, 89:2581-2584; Stratford-Perricadet et al., *J. Clin. Invest.* 1992, 90:626-630; and Rosenfeld et al., *Cell* 1992, 68:143-155. The vector also may contain a selectable marker, for instance β -galactosidase (β -gal) or GFP. In general, preparation of vector containing desired nucleic acid for administration to a subject is in accordance with known procedures as disclosed e.g. in *Molecular Cloning, A Laboratory Manual* (2d ed., Sambrook, Fritsch and Maniatis, Cold Spring Harbor 1989); *Current Protocols in Molecular Biology*, (eds. Ausubel et al., Greene Publ. Assoc., Wiley-Interscience, NY, NY 1992); and *Methods in molecular biology* (ed. EJ Murray, Humana, Clifton, NJ 1991). See also the Examples which follow for exemplary procedures.

Naked nucleic acid also may be administered in accordance with the invention, typically formulated with a pharmaceutically acceptable carrier. For example, a polynucleotide, optionally present as a pharmaceutically acceptable salt, can be present in forms such as suspensions, solutions, emulsions in oily or preferably aqueous vehicles that are preferably sterile and pyrogen-free. Pharmaceutically acceptable salts can be suitably prepared from organic or inorganic bases, such as a sodium or potassium salt, or a salt of a primary, secondary or tertiary amine. The administration solution can suitably contain buffers to adjust the pH of the solution, nonionic materials such as sugars e.g. sucrose to adjust tonicity.

Nucleic acid also may be administered as DNA-liposome or RNA-liposome complexes. Such complexes comprise a mixture of fat particles or lipids which bind

to DNA or RNA to provide a hydrophobic coated delivery vehicle. Suitable liposomes may comprise any of the conventional synthetic or natural phospholipid liposome materials including phospholipids from natural sources such as egg, plant or animal sources such as phosphatidylcholine, phosphatidylethanolamine, 5 phosphatidylglycerol, sphingomyelin, phosphatidylserine or phosphatidylinositol. Synthetic phospholipids also may be used e.g., dimyristoylphosphatidylcholine, dioleoylphosphatidylcholine, dioleoylphosphatidylcholine and corresponding synthetic phosphatidylethanolamines and phosphatidylglycerols. Cholesterol or other sterols, cholesterol hemisuccinate, glycolipids, 1,2-bis(oleoyloxy)-3-(trimethyl 10 ammonio)propane (DOTAP), N-[1-(2,3-dioleoyl)propyl]-N,N,N-trimethylammonium chloride (DOTMA), and other cationic lipids may be incorporated into the liposomes.

Typical subjects to which nucleic acid will be administered for therapeutic application include mammals, particularly primates, especially humans, and subjects 15 for xenotransplant applications such as a primate or swine, especially pigs. For veterinary applications, a wide variety of subjects will be suitable, e.g. livestock such as cattle, sheep, goats, cows, swine and the like; poultry such as chickens, ducks, geese, turkeys and the like; and pets such as dogs and cats. For diagnostic or research applications, a wide variety of mammals will be suitable subjects including rodents 20 (e.g. mice, rats, hamsters), rabbits, primates, and swine such as inbred pigs and the like.

The effective dose of nucleic acid will be a function of the particular expressed protein, the target tissue, the subject (including species, weight, sex, general health, 25 etc.) and the subject's clinical condition. The effective dose of nucleic acid also will be a function of the permeability cocktail, which generally will allow less total viral particles to be delivered. Optimal administration rates for a given protocol of administration can be readily ascertained by those skilled in the art using conventional dosage determination tests. Additionally, frequency of administration for a given 30 therapy can vary, particularly with the time cells containing the exogenous nucleic acid continue to produce the desired polypeptide as will be appreciated by those

skilled in the art. Also, in certain therapies, it may be desirable to employ two or more different proteins to optimize therapeutic results.

Nucleic acid can be administered to selected tissue by a variety of protocols.

- 5 The preferred administration method is perfusion of a liquid formulation of the nucleic acid through a solid organ such as a heart or liver or other solid cell mass such as a solid tumor. It may be preferred to recirculate the perfusate through the organ or cell mass which, among other things, can limit delivery of the nucleic acid to targeted cells. A preferred perfusion protocol is described in the Examples which follow. See
10 also Donahue et al., *Proc Natl Acad Sci USA* 1997, 94:4664-4668 for suitable perfusion procedures.

- If an adenovirus vector is employed, the nucleic acid preferably is administered in a formulation that is essentially (less than about 5 percent by volume)
15 or completely free of red blood cells which can adsorb adenovirus. Krebs solution is a particularly preferred carrier. Preferably the liquid carrier has a low calcium ion concentration as discussed above. However, other solutions, even native bloodstream with physiologic levels of red blood cells and calcium, can be employed and may be suitable for certain applications, e.g. where the primary intervention for enhancing
20 gene transfer is alteration of vascular permeability.

- The concentration of nucleic acid within a liquid carrier can vary, but relatively high concentrations are preferred to provide increase efficiency of nucleic acid uptake as discussed above. More specifically, preferably a viral vector containing
25 nucleic acid for administration is present in a liquid carrier such as Krebs solution at a concentration of about 1×10^8 plaque forming units (pfu)/ml or greater, more preferably a concentration of about or 5×10^8 , still more preferably a concentration of about 1.0×10^9 or 1.5×10^9 pfu/ml or greater.

- 30 Flow rates of a perfusate solution containing nucleic acid also can vary, but are preferably relatively rapid compared to normal blood flow in the delivery vessel to

provide enhanced nucleic acid uptake. For example, a perfusate flow rate of at least about 5, 10, 15, 20, 25, 50 or 75 percent more rapid compared to normal blood flow in the delivery vessel can be employed. In certain applications, even more rapid flow rates will be preferred, e.g. a perfusate flow rate of at least about 100, 200, 300, 400 or
5 500 percent more rapid compared to normal blood flow. Flow rates up to 600, 700, 800, 900 or 1000 percent more rapid compared to normal blood flow rate of a subject also can be suitable for certain applications.

It should be appreciated that a particular application of the invention can be
10 readily optimized, e.g. the optimal permeability agents readily selected as discussed above by simple testing against a control; the use of lowered calcium conditions can be readily assessed by testing relative to a control; perfusate flow rates, perfusate concentrations of nucleic acids and the like also all can be readily optimized by testing relative to a control. Optimal conditions and agents may vary from application to
15 application, e.g. for administration to varying organs or mammals of different species.

Nucleic acid can be administered by perfusion by a variety of strategies. Thus, for instance, for an *in vivo* administration, a catheter delivery protocol can be employed. Such *in situ* administration can be suitably employed in a procedure solely
20 to deliver the nucleic acid, or in conjunction with a separate surgical procedure such as a peripheral cardiac bypass. A preferred delivery catheter and system is disclosed in WO 9918792A1 (see the figures of that patent publication).

Nucleic acid also may be suitably administered by perfusion through a
25 procedure involving extra-corporal circulation such as performed during coronary artery bypass surgery and aortic valve replacement surgery. In such clinical settings, both arterial and venous vessels can be accessed for delivery, collection and possible recirculation of the perfusate formulation thus targeting gene transfer to the heart and minimizing delivery to remote organs or tissues.

Ex vivo perfusion systems also may be employed, particularly in test animals such as a rabbit, rodent, etc. to assess viability and efficiency of specific permeability agents, calcium concentration conditions, cloning vehicles, and perfusion conditions such as flow rate, amount of nucleic acid administered, concentration of nucleic acid in perfusate, etc. An *ex vivo* procedure also can be employed to deliver therapeutic procedures followed by transplanted of the organ into a patient. A suitable *ex vivo* system is shown in figure 2 of WO 9918792A1.

Alternate *ex vivo* methods do not involve removal of an intact organ, but rather removal of selected tissue of an organ followed by reimplanting of the tissue after transformation of the removed cells with the desired exogenous nucleic acid. However, as discussed above, such an approach is generally less preferred for the substantial surgical intervention required and difficulty of effective grafting of genetically modified cells.

Additionally, other therapeutic agents, including non-peptide therapeutics, can be administered together with the gene product of the exogenous nucleic acid. Thus, as one example, nitroglycerine may be co-administered to control vasospasms, or an anticancer agent may be co-administered during treatment of malignant cells.

The invention also provides pharmaceutical kits for treatment of a disorder of a subject. Kits of the invention preferably include a delivery system or device to administer the exogenous nucleic acid such as a catheter as discussed above, an *ex vivo* administration system such as those discussed above, a syringe for injection administration and the like. The kits also will include the nucleic acid to be administered, preferably in a form suitable for administration, e.g. contained in a suitable cloning vehicle such as a viral vector dissolved in desired pharmaceutically acceptable carrier e.g. Krebs solution or other buffered solution that preferably has a low calcium concentration in accordance with the invention and may have other agents such as various buffers, sugars, salts and the like. Alternatively, the nucleic acid may be freeze dried and packaged separately from the fluid carrier. Preferably the

nucleic acid and carrier would be present in the kit in optimal dosage quantities. A pharmaceutical kit of the invention also may contain other therapeutic agents that may be co-administered with the gene product of the exogenous gene product as discussed above.

5

A kit of the invention also preferably contains one or more PDE inhibitor compounds and optional additional, distinct vasculature permeability agents, packaged either separately from or admixed with a liquid formulation to perfuse the one or more agents through a selected area of a subject. The kit also may contain a
10 calcium "washout" formulation to lower the calcium concentration to a desired level in a selected area of a subject. For example, such a "washout" solution could be Krebs solution or other fluid having a low calcium concentration in accordance with the invention and administered prior to administration of the exogenous nucleic acid as discussed above. The one or more permeability agents also may be present in the
15 kit either packaged separately from for later formulation, or pre-formulated with such a low calcium concentration solution for administration prior to delivery of the exogenous nucleic acid. Each of the materials of a kit of the invention, or any composition of the invention that is administered to a subject, particularly a human, should be sterile and otherwise pharmaceutically acceptable. Each of the kit materials
20 intended for administration typically will be stored in a suitable container, preferably each such container hermetically sealed. The kit also suitably may contain written instructions for use and administration of the kit materials.

As discussed above, the invention also provides methods for introducing into a
25 recipient subject transformed donor cells to express a desired gene product in the recipient. Nucleic acid is introduced into the donor cells in accordance with the invention as described herein, i.e. by enhancing vasculature permeability to introduce desired nucleic acid into the cells. Typical recipient subjects for these xenotransplant methods include those subjects disclosed above, i.e. mammals, particularly primates,
30 preferably a human. Donor cells can come from a variety of sources such as from the sources disclosed above. Donor cells from a swine such a pig, or a primate such as a

human, are generally preferred. A solid donor organ containing cells comprising exogenous nucleic acid may be transplanted into the recipient subject. Xenotransplant procedures in general have described in the literature such as in U.S. Patents 5,650,148 and 5,658,564 and the like.

5

All documents mentioned herein are incorporated herein by reference.

The following non-limiting example is illustrative of the invention.

10 General Comments

The following materials and conditions were employed in the following Example 1.

Methods: Quantification of the effect of exposure to vascular permeability
15 mediators was performed using methods reported in the original ROI and patent application as disclosed in WO 9918792A1. This method includes *ex vivo* perfusion of rabbit hearts with the vascular compounds and with Adgal (a recombinant adenovirus encoding E. coli -galactosidase) followed by enzymatic digestion and isolation of the cardiac myocytes, 48 hr cell culture of the myocytes, and X-gal
20 staining to document gene transfer. The specific methods are as follows:

Adenovirus vectors. Adgal contained the E. coli *lac Z* gene driven by the human cytomegalovirus (CMV) immediate early promoter. Virus stocks were expanded as previously described,⁷ aliquoted in small volumes, and stored in
25 phosphate-buffered saline (PBS) with 10% glycerol at -80° C. Viral titers were determined by the average of two plaque assays performed using a traditional method.⁷ Virus stocks were free of replication-competent viruses when tested with a supernatant rescue assay that has the sensitivity to detect one replication-competent virus in 10⁹ recombinant viruses.⁸

30

Langendorff infection. Rabbit ventricular myocytes were infected as previously described⁶. Adult New Zealand white rabbits (2-3 kg, N = 48) received heparin anticoagulation (1000 units IV) prior to pentobarbital (50 mg/kg IV). Each heart was extracted and rinsed twice in ice-cold, modified Krebs buffer containing

5 138.2 mmol/L Na⁺, 5.4 mmol/L K⁺, 1.2 mmol/L Mg²⁺, 1.0 mmol/L Ca²⁺, 144.4 mmol/L Cl⁻, 1.2 mmol/L SO₄²⁻, 1.2 mmol/L H₂PO₄⁻, 20 mmol/L HEPES, and 15 mmol/L glucose, saturated with O₂ at pH 7.4. Next, the aorta was cannulated, and the heart was suspended in an insulated chamber at 35-37° C. During the subsequent experimental manipulations, heart rate, coronary flow and intra-aortic pressure were

10 monitored at 10 minute intervals.

Langendorff perfusion occurred by retrograde flow from the cannula in the ascending aorta to the coronary arteries. Each heart was first perfused with 20 ml Krebs buffer at approximately 30 ml/min. After initial perfusion, the heart was

15 pretreated with one of the following: (1) 15 ml of normal Krebs buffer with 1 x 10⁻⁷ mol/L substance P over 30 sec, (2) 60 ml of normal Krebs buffer with 1 x 10⁻⁹ mol/L VEGF over 2 min, (3) 60 ml of normal Krebs buffer with 9 x 10⁻⁵ mol/L nitroglycerin over 2 min, (4) 60 ml of normal Krebs buffer with 9 x 10⁻⁵ mol/L sodium nitroprusside over 2 min, (5) 60 ml of normal Krebs buffer with 1 x 10⁻⁴ mol/L 8-Br-

20 cGMP over 2 min, (6) 1 x 10⁻⁵ mol/L zaprinast (Sigma), 1 x 10⁻⁵ mol/L sildenafil (Pfizer), or 1 x 10⁻⁶ mol/L T-1032 (Tanabe) for 15 min before 3 x 10⁻¹⁰ mol/L VEGF for 2 minutes.

Following pretreatment, the heart was infected with Krebs buffer containing

25 the pretreatment compound at the stated concentration and Adgal 1 x 10⁸ pfu/ml for 2 minutes. During infection, the perfusate was collected and recirculated, and the flow rate was controlled by a peristaltic pump (Masterflex, Cole-Palmer Co.). At the end of the infection interval, the heart was perfused with non-recirculating, virus-free Krebs buffer for a total Langendorff time of 180 minutes. Following the infection and

washout phases of the experiments, myocytes were isolated and cultured by perfusing with nominally calcium-free Krebs for 5 minutes at 10-15 ml/min, followed by an enzyme solution consisting of Krebs buffer with 25 mol/L Ca^{2+} , 1 mg/ml collagenase B (Boehringer-Mannheim, Indianapolis, IN), 0.1 mg/ml protease (fraction XIV, Sigma, St. Louis, MO), 60 mmol/L taurine, 8.0 mmol/L glutamic acid, and 2.0 mmol/L d,l-carnitine. After digestion, the ventricles were excised, minced, and agitated by repeated suction through a Pasteur pipette. The resulting cell suspension was strained through a 200 m nylon mesh filter. The ventricles were uniformly digested, and the residue remaining on the filter was from the valvular and tendon structures, with only minimal myocardium. Calcium was gradually repleted in four steps before the cells were added to medium 199 (Mediatech, Herndon, VA) supplemented with 5.0 mmol/L creatine, 5.0 mmol/L d,l-carnitine, 5.0 mmol/L taurine, 100 u/ml penicillin and 0.1 mg/ml streptomycin, and placed in laminin-coated dishes, in a 37°C incubator. The myocardial digestion and number of viable cells with each isolation, quantified by counting morphologically normal myocytes, were not affected by any of the experimental manipulations.

In separate control experiments, hearts underwent a sham infection (N = 2). None of the cells from these experiments stained positive for -galactosidase activity. Similar negative control data in cultured adult rat ventricular myocytes have been reported.⁷

The animals used in this study were maintained in accordance with the guiding principles of the American Physiological Society regarding experimental animals. The experimental protocol was approved by the Institutional Animal Care and Use Committee at the Johns Hopkins University.

Reporter Gene Assays and Data Analysis. Forty-eight hours after exposure to Adgal, the myocytes were fixed in 0.05% glutaraldehyde for 5 minutes at room temperature. The cells were then washed three times in PBS and stained overnight at 37°C in PBS containing 1.0 mg/ml 5-bromo, 4-chloro, 3-indolyl--D-galactopyranoside (X-gal), 15 mmol/L potassium ferricyanide, 15 mmol/L potassium ferrocyanide and 1 mmol/L MgCl_2 . Four hundred cells were counted for each

experiment. Myocyte survival at 48 hours did not vary with changes in the virus concentration or experimental protocol.

Statistical Analysis. Unless otherwise stated, all experiments were performed in triplicate and the data are presented as mean \pm s.d. Statistical significance was determined at the 5% level using the unpaired Student's *t*-test.

Example 1:

Administration of Substance P and VEGF. Treatment of the *ex vivo* perfused heart with either 1×10^{-7} M Substance P or 1×10^{-9} M VEGF prior to infection with Adgal results in a significant increase in the percentage infected cells (Fig. 1). These agents increase microvascular permeability faster than either bradykinin or serotonin, and are able to achieve the same enhancement of infection with 2 min of pretreatment that requires 15 min of serotonin exposure.

Treatment with nitroglycerin, nitroprusside, or 8-Br-cGMP. The intracellular pathways that mediate increased vascular permeability after VEGF or Substance P exposure include production of nitric oxide and cGMP.¹⁻⁵ Exogenous administration of nitroglycerin or nitroprusside causes an increase in local nitric oxide. Likewise, 8-Br-cGMP is a cGMP analog that is metabolized more slowly than cGMP, causing increases in the effective concentration of cGMP within the cells. Exposure of the *ex vivo* perfused heart to any of these agents prior to Adgal replicates the increase in infection efficiency caused by VEGF or Substance P administration (Fig. 2).

Sildenafil, zaprinast or T-1032 pretreatment before VEGF exposure. Vascular endothelial cells metabolize cGMP using phosphodiesterase-5 (PDE-5).⁵⁻⁶ Administration of PDE-5 inhibitors was performed prior to VEGF and Adgal exposure to evaluate the effect on infection efficiency. Like the results with 8-Br-cGMP, inhibition of PDE-5 followed by VEGF treatment increased the percentage of

infected cells (Fig. 3). No change was seen in the absence of VEGF, indicating that the effect was indeed from PDE-5 inhibition and not a non-specific finding.

These data show increased gene delivery to the intact myocardium.

- 5 Furthermore, increasing the intracellular concentration of the components of the signalling pathway responsible for the permeability effect improves gene delivery. Taking advantage of this understanding of the intracellular signalling pathways responsible for increasing microvascular permeability, it is shown that use of a PDE-5 inhibitor can particularly delivery effects. Since the exposure time required for
- 10 VEGF, substance P, 8-Br-cGMP, nitroglycerin or nitroprusside is only 2 minutes and the PDE-5 inhibitors can be given orally or intravenously before the procedure, the current innovations dramatically reduce the pretreatment time before gene delivery.

- Results also are disclosed in Nagata et al., *J. Mol Cell Cardio*, 33: 575-580
- 15 (2001), incorporated herein by reference.

The following specific references, also incorporated by reference, are indicated in the examples and discussion above by a number in parentheses.

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This invention has been described in detail with reference to preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements within the spirit and scope of the invention.